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<p>(21) International Application Number: PCT/US94/10264 (22) International Filing Date: 13 September 1994 (13.09.94)  (30) Priority Data: 08/122,230 17 September 1993 (17.09.93) US 08/122,827 17 September 1993 (17.09.93) US 08/162,827 3 December 1993 (03.12.93) US 08/172,331 22 December 1993 (22.12.93) US  (71) Applicants: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK). NOVO NORDISK BIOTECH, INC. [US/US]; 1445 Drew Avenue, Davis, CA 95616-4880 (US).  (72) Inventors: WAHLEITHNER, Jill, Angela; 1718 Tea Place, Davis, CA 95616 (US). CHRISTENSEN, Bjørn, Eggert; Dronninggaards A11 32, DK-2840 Holte (DK). SCHNEIDER, Palle; Rydtoften 43, DK-2750 Ballerup (DK).  (74) Agents: ZELSON, Steve, T. et al.; Novo Nordisk of North America, Inc., Suite 6400, 405 Lexington Avenue, New York, NY 10174 (US).</p>	<p>(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, NO, NZ, PL, RO, RU, SD, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: PURIFIED pH NEUTRAL RHIZOCTONIA LACCASES AND NUCLEIC ACIDS ENCODING SAME</p>		
<p>(57) Abstract</p> <p>The present invention relates to isolated nucleic acid fragments containing a sequence encoding a <i>Rhizoctonia solani</i> laccase having optimum activity at a neutral or basic pH, and the laccase proteins encoded thereby.</p>		

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PURIFIED PH NEUTRAL RHIZOCTONIA LACCASES AND NUCLEIC  
ACIDS ENCODING SAME

5

Related Applications

This application is a continuation-in-part of co-  
pending U.S. Serial Nos. 08/122,230, 08/122,827, and  
08/162,827, the contents of which are incorporated by  
10 reference in their entirety.

Field of the Invention

The present invention relates to isolated nucleic acid  
fragments encoding a fungal oxidoreductase enzyme and the  
15 purified enzymes produced thereby. More particularly, the  
invention relates to nucleic acid fragments encoding a  
phenol oxidase, specifically a laccase, which functions at  
a neutral pH.

20 Background of the Invention

Laccases (benzenediol:oxygen oxidoreductases) are  
multi-copper containing enzymes that catalyze the oxidation  
of phenolics. Laccase-mediated oxidations result in the  
production of aryloxy-radical intermediates from suitable  
25 phenolic substrate; the ultimate coupling of the  
intermediates so produced provides a combination of dimeric,  
oligomeric, and polymeric reaction products. Such reactions  
are important in nature in biosynthetic pathways which lead  
to the formation of melanin, alkaloids, toxins, lignins, and  
30 humic acids. Laccases are produced by a wide variety of  
fungi, including ascomycetes such as *Aspergillus*,  
*Neurospora*, and *Podospora*, the deuteromycete *Botrytis*, and

basidiomycetes such as *Collybia*, *Fomes*, *Lentinus*, *Pleurotus*,  
*Trametes*, and perfect forms of *Rhizoctonia*. Laccase  
exhibits a wide range of substrate specificity, and each  
different fungal laccase usually differs only quantitatively  
5 from others in its ability to oxidize phenolic substrates.  
Because of the substrate diversity, laccases generally have  
found many potential industrial applications. Among these  
are lignin modification, paper strengthening, dye transfer  
inhibition in detergents, phenol polymerization, juice  
10 manufacture, phenol resin production, and waste water  
treatment.

Although the catalytic capabilities are similar,  
laccases made by different fungal species do have different  
temperature and pH optima, and these may also differ  
15 depending on the specific substrate. A number of these  
fungal laccases have been isolated, and the genes for  
several of these have been cloned. For example, Choi et  
al. (*Mol. Plant-Microbe Interactions* 5: 119-128, 1992)  
describe the molecular characterization and cloning of the  
20 gene encoding the laccase of the chestnut blight fungus,  
*Cryphonectria parasitica*. Kojima et al. (*J. Biol. Chem.*  
265: 15224-15230, 1990; JP 2-238885) provide a description  
of two allelic forms of the laccase of the white-rot  
basidiomycete *Coriolus hirsutus*. Germann and Lerch  
25 (*Experientia* 41: 801, 1985; *PNAS USA* 83: 8854-8858, 1986)  
have reported the cloning and partial sequencing of the  
*Neurospora crassa* laccase gene. Saloheimo et al. (*J. Gen.*  
*Microbiol.* 137: 1537-1544, 1985; WO 92/01046) have  
disclosed a structural analysis of the laccase gene from the  
30 fungus *Phlebia radiata*. However, virtually all of the  
known fungal laccases function best at acidic pHs (e.g.,  
between pH 3.0 and 6.0), and are typically inactive at

neutral or basic pHs. Since a number of the aforestated potential industrial methods are preferentially conducted at neutral or basic pH, most fungal laccases perform poorly in such methods. Thus, the available fungal laccases are  
5 inadequate for application in a number of important commercial methods.

An exception to this rule is the extracellular laccase produced by certain species of *Rhizoctonia*. Bollag et al. have reported a laccase with a pH optimum of about 7.0  
10 produced by *Rhizoctonia praticola*. A laccase of this type would be far more useful in industrial methods requiring neutral pH than previously known laccases. However, the *R. praticola* enzyme was neither purified nor further characterized, nor, to date, has any other laccase having  
15 this trait been purified or characterized. Moreover, although other laccase genes have been isolated, as described above, these have been genes encoding enzymes which function best at acidic pH. Recombinant production and commercially adequate yields of a pH neutral or basic  
20 laccase have thus been unattainable due to the fact that neither the enzyme per se nor the laccase gene encoding such an enzyme has previously been isolated and/or purified and sequenced. The present invention now provides a solution to each of these problems.

25

#### Summary of the Invention

The present invention relates to an isolated nucleic acid fragment comprising a nucleic acid sequence encoding a *Rhizoctonia* laccase which functions optimally at a pH  
30 between 6.0 to 8.5. By "functioning optimally" is meant that the enzyme exhibits significant (i.e., at least about 30% of maximum, preferably at least about 50%, and most

preferably from 50% to maximum) activity within the pH range of between about 6.0-8.5, as determined by activity in one or more standard laccase assays for substrates such as the syringaldazine, ABTS, 2,6-dimethoxyphenol, or 4  
5 antiaminopyrine + N-ethyl-N-sulfobutyl-m-toluidine. A preferred substrate for the laccases of the present invention is syringaldazine. In a preferred embodiment, the laccase is a *Rhizoctonia solani* laccase. The invention also relates to a substantially pure laccase encoded by the novel  
10 nucleic acid sequence. By "substantially pure" is meant a laccase which is essentially (i.e., ≥90%) free of other non-laccase proteins.

In order to facilitate production of the novel laccase, the invention also provides vectors and host cells  
15 comprising the claimed nucleic acid fragment, which vectors and host cells are useful in recombinant production of the laccase. The nucleic acid fragment is operably linked to transcription and translation signals capable of directing expression of the laccase protein in the host cell of  
20 choice. A preferred host cell is a fungal cell, most preferably of the genus *Aspergillus*. Recombinant production of the laccase of the invention is achieved by culturing a host cell transformed or transfected with the nucleic acid fragment of the invention, or progeny thereof, under  
25 conditions suitable for expression of the laccase protein, and recovering the laccase protein from the culture.

The laccases of the present invention are useful in a number of industrial processes in which oxidation of phenolics is required. These processes include lignin  
30 manipulation, juice manufacture, phenol polymerization and phenol resin production. In a preferred embodiment, the

enzyme of the invention is used in a process requiring a neutral or somewhat basic pH for greatest efficiency.

#### Brief Description of the Figures

5        Figure 1 illustrates the nucleotide and amino acid sequence of RSlac1. Lower case letters in the nucleotide sequence indicate the position of introns.

         Figure 2 illustrates the nucleotide and amino acid sequence of RSlac2. Lower case letters in the nucleotide  
10        sequence indicate the position of introns.

         Figure 3 illustrates a restriction map of the plasmid PMWR-1.

         Figure 4 illustrates the nucleotide and amino acid sequence of the translated region of RSlac3.

15        Figure 5 illustrates the syringaldazine oxidase activity of RSlac1 (90mM buffer, 20  $\mu$ M syringaldazine, 20°C).

         Figure 6 illustrates the syringaldazine oxidase activity of RSlac2 (93mM buffer, 20  $\mu$ M syringaldazine,  
20        20°C).

#### Detailed Description of the Invention

         Certain species of the genus *Rhizoctonia* have been reported as producing laccase; therefore, an initial search focused on identifying the presence of these enzymes in  
25        various *Rhizoctonia solani* isolates. Samples are cultured and the supernatants periodically analyzed for the presence of laccase by the ABTS method, described below. Laccase is observed in all the *Rhizoctonia* cultures. Harvested  
         laccases are electrophoretically separated and stained with  
30        ABTS. One isolate, RS22, produces a laccase with a basic pI, and is selected for further study.

The remaining studies focus on purification and characterization of the enzyme from RS22. Briefly, the fermentation broth is filtered and concentrated by UF with a membrane cut off of about 10,000. A first ion exchange  
5 chromatography step is conducted at pH 4.5 in acetate buffer, with step elution using NaCl. The eluate is then ultrafiltered and rechromatographed, and eluted with a NaCl gradient. Active fractions are pooled for further study.

The intact protein thus isolated and purified  
10 (hereinafter referred to as RSlac3) is first subjected to partial sequencing, and the N-terminal sequence obtained is as follows:

AVRNYKFDIKNVNVAPDGFQRPIVSV (SEQ. ID. NO.: 5)

The protein is further subjected to digestion with a  
15 lysine- or glutamic-acid specific protease, and additional peptides obtained from the protein have the following sequences, which can be aligned with sequences in *Coriolus hirsutus*:

Peptide 1:

20 SQYVDGLRGPLVIYDPDDDH (SEQ. ID. NO: 6)

Peptide 2:

GLALVFAEAPSQIRQGVQSVQPDDA (SEQ. ID. NO.: 7)

Peptide 3:

SRVBVBASTVVMLEBWYHTPAXVLE (SEQ. ID. NO. 8)

25 Peptide 4:

SLGPTPNYVNPXIRDVVRVGTTVV (SEQ. ID. NO. 9)

The following peptides are also found, but do not correspond to *Coriolus* sequences

Peptide 5:

30 IRYVGGPVAVX(N?)RSVI (SEQ. ID. NO.: 10)

Peptide 6:

ILANPA (SEQ. ID. NO.: 11)



Peptide 7:

YEAPSLPT (SEQ. ID. NO.: 12)

In the above sequences, B designates a residue which is either aspartic acid or asparagine, and X designates  
5 unidentified residues.

In order to initiate screening for a *Rhizoctonia* laccase gene, an *R. solani* genomic library is prepared. Total DNA is partially digested with restriction enzyme Sau3A, and electrophoresed in an agarose gel to isolate DNA  
10 fragments between 8 and 21 kb in size. The fractionated fragments are ligated to  $\lambda$  phage EMBL3 arms with BamHI ends, and the resulting phage packaged in vitro. These phage are used as a library to create a library of 170,000 plaques in *E. coli* and amplified 100-fold for future use.

15 In order to develop probes for isolation of the *R. solani* laccase gene, the protein sequences of five known laccases are analyzed to determine consensus sequences, and two degenerate oligonucleotides constructed based on observed consensus sequences (Choi et al. *supra*; Germann and  
20 Lerch, *supra*; Saloheimo et al, *supra*, Kojima et al, *supra*). These oligos are mixed with *R. solani* genomic DNA and a DNA fragment of 220 nucleotide fragment is amplified using a taq polymerase chain reaction(PCR). The 220-nucleotide fragment is then cloned into plasmid vector.

25 The PCR fragment is used as a probe to screen 25,000 plaques from the amplified genomic library. Positive clones from this screen fall into two classes that are subsequently shown, by DNA sequence analysis, to code for two different laccase genes, *RSlac1* and *RSlac2*. The nucleotide sequence  
30 for each of these genes (SEQ ID. NOS.: 1 and 3), and the predicted amino acid sequence for each protein (SEQ. ID. NOS.: 2 and 4), are presented in, respectively, Figures 1

and 2. The homology between the two sequences is approximately 63%. Compared to known laccase sequences from *Coriolus hirsutus*, *Phlebia radiata*, *Aspergillus nidulans*, *Cryphonectria parasitica*, and *Neurospora crassa*, the RS  
5 laccases show between about 30-40% homology. Each of the two coding sequences is cloned into an expression vector operably linked to *Aspergillus oryzae* taka-amylase transcription and translation signals (See Figure 3). Each of the two laccase expression vectors is transformed into an  
10 *Aspergillus oryzae* and *Aspergillus niger* host cell, and the host cells screened for the presence of laccase.

For isolation of the RSlac3 gene, polyA RNA is purified from *R. solani* mycelia grown in the presence of anisidine. The RNA is used as a template for cDNA synthesis. The cDNA  
15 is fractionated and fragments between 1.7-3.5 kb collected, and a cDNA library created by cloning the fractionated DNA into a yeast vector. 3000 transformants from this library are screened on ABTS. After 24 hours, a single colony appears positive. The plasmid from the colony is isolated  
20 and the insert sequenced. Portions of the predicted amino acid sequence correspond with the sequences of the fragments obtained from RS 22, described supra. The complete nucleotide and amino acid sequences are depicted in Figure 4, and in SEQ. ID. NOS.: 13 and 14, respectively. RSlac3  
25 shows 48% homology with RSlac1 and 50% homology with RSlac2. RSlac3 also shows 48% homology with the *Coriolus hirsutus* laccase gene.

According to the invention, a *Rhizoctonia* gene encoding a pH neutral or basic laccase can be obtained by  
30 methods described above, or any alternative methods known in the art, using the information provided herein. The gene can be expressed, in active form, using an expression

vector. A useful expression vector contains an element that permits stable integration of the vector into the host cell genome or autonomous replication of the vector in a host cell independent of the genome of the host cell, and  
5 preferably one or more phenotypic markers which permit easy selection of transformed host cells. The expression vector may also include control sequences encoding a promoter, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes. To  
10 permit the secretion of the expressed protein, nucleotides encoding a signal sequence may be inserted prior to the coding sequence of the gene. For expression under the direction of control sequences, a laccase gene to be treated according to the invention is operably linked to the  
15 control sequences in the proper reading frame. Promoter sequences that can be incorporated into plasmid vectors, and which can direct the transcription of the laccase gene, include but are not limited to the prokaryotic  $\beta$ -lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad.  
20 Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references can also be found in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; and in Sambrook et al., Molecular Cloning, 1989.

25

The expression vector carrying the DNA construct of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will typically depend on the host cell into which it  
30 is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is

independent of chromosomal replication, e.g. a plasmid, or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host  
5 cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may  
10 be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA construct of the invention,  
15 especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis*  $\alpha$ -amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the  
20 promoters of the *Bacillus amyloliquefaciens*  $\alpha$ -amylase (*amyQ*), or the promoters of the *Bacillus subtilis* *xylA* and *xylB* genes. In a yeast host, a useful promoter is the *eno-1* promoter. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding A.  
25 *oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral  $\alpha$ -amylase, *A. niger* acid stable  $\alpha$ -amylase, *A. niger* or *A. awamsii* glucoamylase (*gluA*), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase. Preferred  
30 are the TAKA-amylase and *gluA* promoters.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the laccase of the invention.

5 Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter. The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19,  
10 pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. li-*  
15 *cheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Examples of *Aspergillus* selection markers include *amdS*, *pyrG*, *argB*, *niaD* and *sc*, a marker giving rise to hygromycin resistance. Preferred for use in an  
20 *Aspergillus* host cell are the *amdS* and *pyrG* markers of *A. nidulans* or *A. oryzae*. A frequently used mammalian marker is the dihydrofolate reductase (DHFR) gene. Furthermore, selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

25

It is generally preferred that the expression is extracellular. The laccases of the present invention may thus comprise a preregion permitting secretion of the expressed protein into the culture medium. If desirable,  
30 this preregion may be native to the laccase of the invention or substituted with a different preregion or signal sequence, conveniently accomplished by substitution of the

DNA sequences encoding the respective preregions. For example, the preregion may be derived from a glucoamylase or an amylase gene from an *Aspergillus* species, an amylase gene from a *Bacillus* species, a lipase or proteinase gene from

5 *Rhizomucor miehei*, the gene for the  $\alpha$ -factor from *Saccharomyces cerevisiae* or the calf prochymosin gene. Particularly preferred, when the host is a fungal cell, is the preregion for *A. oryzae* TAKA amylase, *A. niger* neutral amylase, the maltogenic amylase form *Bacillus* NCIB 11837, *B.*

10 *stearothermophilus*  $\alpha$ -amylase, or *Bacillus licheniformis* subtilisin. An effective signal sequence is the *A. oryzae* TAKA amylase signal, the *Rhizomucor miehei* aspartic proteinase signal and the *Rhizomucor miehei* lipase signal.

15 The procedures used to ligate the DNA construct of the invention, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance,

20 Sambrook et al. Molecular Cloning, 1989).

The cell of the invention either comprising a DNA construct or an expression vector of the invention as defined above is advantageously used as a host cell in the

25 recombinant production of a enzyme of the invention. The cell may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more

30 likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed

according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

5

The host cell may be selected from prokaryotic cells, such as bacterial cells. Examples of suitable bacteria are gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus*  
10 *stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E.coli*. The  
15 transformation of the bacteria may for instance be effected by protoplast transformation or by using competent cells in a manner known per se.

The host cell may also be a eukaryote, such as mammalian cells, insect cells, plant cells or preferably  
20 fungal cells, including yeast and filamentous fungi. For example, useful mammalian cells include CHO or COS cells. A yeast host cell may be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. Useful filamentous fungi may be selected from a  
25 species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Alternatively, a strain of a *Fusarium* species, e.g. *F. oxysporum*, can be used as a host cell. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per  
30 se. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023. A suitable method of

transforming *Fusarium* species is described by Malardier et al., 1989.

The present invention thus provides a method of producing a recombinant laccase of the invention, which  
5 method comprises cultivating a host cell as described above under conditions conducive to the production of the enzyme and recovering the enzyme from the cells and/or culture medium. The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in  
10 question and obtaining expression of the laccase of the invention. Suitable media are available from commercial suppliers or may be prepared according to published formulae (e.g. in catalogues of the American Type Culture Collection).

15 The resulting enzyme may be recovered from the medium by conventional procedures including separating the cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, followed  
20 by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like. Preferably, the isolated protein is about 90% pure as determined by SDS-PAGE, purity being most important in food,  
25 juice or detergent applications.

In a particularly preferred embodiment, the expression of laccase is achieved in a fungal host cell, such as *Aspergillus*. As described in detail in the following examples, the laccase gene is ligated into a plasmid  
30 containing the *Aspergillus oryzae* TAKA  $\alpha$ -amylase promoter, and the *Aspergillus nidulans* *amdS* selectable marker. Alternatively, the *amdS* may be on a separate plasmid and



used in co-transformation. The plasmid (or plasmids) is used to transform an *Aspergillus* species host cell, such as *A. oryzae* or *A. niger* in accordance with methods described in Yelton et al. (PNAS USA 81: 1470-1474, 1984).

5        Those skilled in the art will recognize that the invention is not limited to use of the nucleic acid fragments specifically disclosed herein, for example, in Figures 1 and 2. It will be apparent that the invention also encompasses those nucleotide sequences that encode the  
10 same amino acid sequences as depicted in Figures 1, 2 and 3, but which differ from those specifically depicted nucleotide sequences by virtue of the degeneracy of the genetic code. In addition, the invention also encompasses other nucleotide fragments, and the proteins encoded thereby, which encode  
15 laccase proteins having substantially the same pH optimum as those of *Rhizoctonia solani*, and which show a significant level of homology with the *Rhizoctonia solani* amino acid sequence. For example, the present data show that more than one species of *Rhizoctonia* produces a laccase with the  
20 desired pH profile; it is therefore expected that other *Rhizoctonia* species also produce similar laccases and therefore, using the technology described herein, can be used as a source for genes within the scope of the claimed invention. As also shown in the present examples, not only  
25 is there more than one nucleotide and amino acid sequence that encodes a laccase with the required characteristics, there is also considerable variation tolerated within the sequence while still producing a functional enzyme. Therefore, the invention also encompasses any variant  
30 nucleotide sequence, and the protein encoded thereby, which protein retains at least about an 80% homology with one or the other of the amino acid sequences depicted in Figures 1,

2 and 3, and retains both the laccase and pH optimum activity of the sequences described herein. In particular, variants which retain a high level (i.e.,  $\geq 80\%$ ) of homology at highly conserved regions of the *Rhizoctonia* laccase are contemplated. Such regions are identified as residues 458-469 in RSLAC1, and 478-489 in RSLAC2; and residues 131-144 in RSLACI and 132-145 in RSLAC2.

Useful variants within the categories defined above include, for example, ones in which conservative amino acid substitutions have been made, which substitutions do not significantly affect the activity of the protein. By conservative substitution is meant that amino acids of the same class may be substituted by any other of that class. For example, the nonpolar aliphatic residues Ala, Val, Leu, and Ile may be interchanged, as may be the basic residues Lys and Arg, or the acidic residues Asp and Glu. Similarly, Ser and Thr are conservative substitutions for each other, as are Asn and Gln. It will be apparent to the skilled artisan that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active enzyme. Retention of the desired activity can readily be determined by conducting a standard ABTS oxidation method in 0.1M sodium phosphate at pH 7.0.

The protein can be used in number of different industrial processes; although the enzyme is also functional to some extent at lower pH, the *R. solani* laccase is most beneficially used in processes that are usually conducted at a neutral or alkaline pH, since other laccases are not active in this pH range. These processes include polymerization of lignin, both Kraft and lignosulfates, in solution, in order to produce a lignin with a higher molecular weight. A neutral/alkaline laccase is a

particular advantage in that Kraft lignin is more soluble at higher pHs. Such methods are described in, for example, Jin et al., *Holzforschung* 45(6): 467-468, 1991; US Patent No. 4,432,921; EP 0 275 544; PCT/DK93/00217, 1992.

5       The laccase of the present invention can also be used for in-situ depolymerization of lignin in Kraft pulp, thereby producing a pulp with lower lignin content. This use of laccase is an improvement over the current use of chlorine for depolymerization of lignin, which leads to the  
10 production of chlorinated aromatic compounds, which are an environmentally undesirable by-product of paper mills. Such uses are described in, for example, Current opinion in Biotechnology 3: 261-266, 1992; J. Biotechnol. 25: 333-339, 1992; Hiroi et al., *Svensk papperstidning* 5: 162-166, 1976.  
15 Since the environment in a paper mill is typically alkaline, the present laccase is more useful for this purpose than other known laccases, which function best under acidic conditions.

Oxidation of dyes and other chromophoric compounds  
20 leads to decolorization of the compounds. Laccase can be used for this purpose, which can be particularly advantageous in a situation in which a dye transfer between fabrics is undesirable, e.g., in the textile industry and in the detergent industry. Methods for dye transfer inhibition  
25 and dye oxidation can be found in WO 92/01406, WO 92/18683, EP 0495836 and Calvo, *Mededelingen van de Faculteit Landbouw-wetenschappen/Rijksuniversitet Gent*.56: 1565-1567, 1991.

The present laccase can also be used for the  
30 polymerization of phenolic compounds present in liquids. An example of such utility is the treatment of juices, such as apple juice, so that the laccase will accelerate a

precipitation of the phenolic compounds present in the juice, thereby producing a more stable juice. Such applications have been described in Stutz, Fruit processing 7/93, 248-252, 1993; Maier et al., Dt. Lebensmittel-  
5 rindschau 86(5): 137-142, 1990; Dietrich et al., Fluss. Obst 57(2): 67-73, 1990. The invention is further illustrated by the following non-limiting examples.

#### EXAMPLES

##### 1. Purification and characterization of *R. solani* laccase

- 10 Individual isolates of *R. solani* cultured on potato dextrose agar (Difco) are examined for laccase enzyme formation by transferring a small piece of agar containing vigorous growth to 100 ml CFM ( 24.0 g potato dextrose broth, 3.0 g yeast extract, 1.0 ml Microelement solution  
15 [0.80 g  $\text{KH}_2\text{PO}_4$ , 0.64 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.11 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.80 g  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.15 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , distilled water to 1000 ml], distilled water to 1000 ml) in a 500 ml shake flask. Incubation is at room temperature, at 200 rpm on an orbital shaker.
- 20 Samples are harvested at 50, 74, 122 and 170 hours, centrifuged and the clear supernatant analyzed for laccase with its ABTS (ABTS= 2,2'-azinobis (3 ethylbenzothiazoline-6-sulfonic acid). The analysis is carried out by adding 200  $\mu\text{l}$  of 2mM ABTS in 0.1 M phosphate buffer, pH 7, and  
25 observing the change in absorbance at 418 nm after 30 minutes incubation at room temperature (approximately 23-25° C). This method is modified from a peroxidase analysis method described by Pütter and Becker (Peroxidases, in: Bergmeyer, H.U.(ed.), Methods of Enzymatic Analysis, 3rd  
30 ed., Vol.III, pp.286-293, 1983)

Each of the laccases harvested at 172 hours is electrophoretically separated and stained with ABTS as

chromogen. Several distinct patterns emerge; the strain RS 22 is shown to produce a laccase having a basic pI, and is chosen for further characterization.

Laccase activity is also determinable by the  
5 syringaldazine method. Laccase catalyzes the oxidation of syringaldazine to tetramethoxy azo bis-methylene quinone under aerobic conditions, with a change of color from yellow to violet. 3000  $\mu$ l of 25 mM acetate buffer (containing 10mg/l cuprisulfate, 5 H<sub>2</sub>O) at pH 5.5, 30°C, is mixed in a 1  
10 cm cuvette with 225  $\mu$ l 0.28 mM syringaldazine (5mg solubilized in 25 ml ethanol and adjusted to 50 ml with demineralized water). The mixture is then mixed with 100  $\mu$ l of a laccase dilution (diluted in acetate buffer so that the increase in absorbance( $\Delta$ OD) is within the range of 0.1-0.6).  
15 The reaction mixture is placed in a 30°C thermostated spectrophotometer and the reaction is followed at 530 nm for 10 to 70 seconds from the addition of laccase. The activity of the enzyme is calculated as  $\Delta$ OD/minute x 0.677 x dilution factor, and is expressed as LACU.

20 For purification of the *Rhizoctonia* laccase, 2.1 liter of culture medium with a LACU activity of 0.19 LACU/ml is filtered through a 10  $\mu$ m filter and concentrated to 230 ml by ultrafiltration using a Filtron Minisette OMEGA membrane with a cutoff value of 10 kDa. The pH of the sample is 5.3  
25 and the activity of the concentrated sample is determined to be 3.34 LACU/ml.

After pH adjustment to 4.5 and filtration due to slight precipitation, the sample is applied to a 40 ml S Sepharose Fast Flow column equilibrated with 20mM acetate buffer at pH  
30 4.5 (buffer A). The column is washed in buffer A and eluted with buffer A containing 1 M NaCl. Active fractions are collected and pooled. This active pool is concentrated and

buffer exchanged to buffer A using an Amicon ultrafiltration unit equipped with a Diaflo YM10 membrane. This sample is rechromatographed on a 5 ml S Sepharose High Performance column using the method described above except that elution is carried out with a linear gradient over 30 column volumes from buffer A to buffer A containing 0.5 M NaCl. The fractions from this purification exhibiting highest activity are pooled. Approximately 45 mg laccase are obtained, when protein concentration is estimated by one absorption unit at A280 nm equal to 1mg/ml. The protein is >90% pure as judged by SDS-PAGE. The molecular weight estimated by SDS-PAGE is approximately 67 kDa. The specific activity of the purified protein is 1 LACU/mg. The pH profile of the purified protein, using syringaldazine as substrate is show in Table 1, below.

Table 1.

pH	5	6	7	8
% activity	0.5	31	100	59

For sequencing of the protein, peptides are generated using wither a lysine-specific protease from *Achromobacter* (*Achromobacter* protease I) or a glutamic acid specific protease from *Bacillus licheniformes*. The peptides are purified by reverse phase HPLC employing linear gradients of 80% 2-propanol containing 0.08% aqueous TFA (solvent B) in 0.1% aqueous TFA (solvent A).

N-terminal amino acid sequence analysis of the intact protein and of purified peptides are carried out in an Applied Biosystems 473A protein sequencer according to the manufacturer's instructions. Initial partial sequencing of

the isolated protein yields the following N-terminal sequence:

AVRNYKFDIKNVNVAPDGFQRPIVSV (SEQ. ID. NO.: 5)

The protein is then digested with either a lysine- or glutamic-acid specific protease, and following additional peptides identified. Peptides 1-4 can be aligned with sequences in the laccase of *Coriolus hirsutus*:

Peptide 1:

SQYVDGLRGPLVIYDPDDDH (SEQ. ID. NO: 6)

10 Peptide 2:

GLALVFAEAPSQIRQGVQSVQPDDA (SEQ. ID. NO.: 7)

Peptide 3:

SRVBVBASTVVMLEBWYHTPAXVLE (SEQ. ID. NO. 8)

Peptide 4:

15 SLGPTPNYVNPXIRDVVRVGGTTVV (SEQ. ID. NO. 9)

Peptide 5:

IRYVGGPAVX(N?)RSVI (SEQ. ID. NO.: 10)

Peptide 6:

ILANPA (SEQ. ID. NO.: 11)

20 Peptide 7:

YEAPSLPT (SEQ. ID. NO.: 12)

An X in the above sequences designates an unidentified residue, and B represents a residue which is either aspartic acid or asparagine.

25

## 2. Isolation of *R. solani* laccase gene

A study of the known amino acid sequences of fungal laccases obtained from non-*Rhizoctonia* species (Choi et al., *supra*; German et al., *supra*; Saloheimo et al. *supra*; and  
30 Kojima et al, *supra*) is conducted to determine the presence of consensus sequences among them. Two regions of high identity, IHWHGFFQ and TFWYHSH, are found near the amino

terminal third of the protein. Based on these consensus sequences and the corresponding DNA sequences, three degenerate oligonucleotides, O-lac2

[TGG/AAAGACCATA/GGTGTCTG/AGTA/G], its complement O-lac2r, and  
5 O-lac3[ATCCAT/CTGGCAT/CGGG/CA/TTCTTCCAG/A], are synthesized using an Applied Biosystems 394 DNA/RNA synthesizer.

The synthesized oligos are used in a polymerase chain reaction (PCR) to screen *Rhizoctonia solani* genomic DNA for a laccase gene or fragment thereof. For amplifications of  
10 genomic DNA, 0.5 µg of genomic DNA is incubated with 1µM of each primer, 200µM of dNTPs, and 1 U taq polymerase (Boehringer Mannheim) in [10 mM Tris-Cl, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 1 mg/ml gelatine; pH 8.3]. The reactions are incubated for 1x5 minutes at 95°C, 30x[1 minute at 95°C, 1 minute at  
15 50-60°C, 1 minute at 72°C], and 1x5 minutes at 72°C. The PCR reactions amplify a DNA fragment of 220 nucleotides. The PCR product is cloned, according to manufacturer's directions, into the TA cloning vector (Invitrogen Corp.). Characterization of the PCR product by DNA sequencing of  
20 individual clones distinguishes two separate laccase genes designated RSlac1 and RSlac2.

To prepare a *R. solani* genomic library, *R. solani* DNA is partially digested with restriction enzyme Sau3A, and electrophoresed through a 0.8% Sea Plaque Agarose (FMC  
25 Bioproducts) in a Tris/Acetate/EDTA buffer to isolate those DNA fragments between 8.0 and 21 kb in size. The gel fractionated fragments are further purified with Beta-Agarase (New England Biolabs) according to manufacturer's instruction, and then ligated to lambda phage EMBL3 arms  
30 with BamHI ends. The resulting phages are packaged in vitro using Gigapack II packaging extract (Stratagene). 25 ml of TB media+0.2% maltose and 10 MgSO<sub>4</sub> is inoculated into a 50 µl



aliquot of an overnight culture of *E. coli* K802 (*supE*, *hsdR*,  
*gal*, *metB*) and incubated at 37°C with shaking until the  
A600=0.5. 25 µl of a 1:10 and 1:50 dilution of the packaged  
phage are mixed with 250 µl of the K802 cells, and incubated  
5 for 20 minutes at 37°C. To each dilution, 5 µl of melted  
top agar at 48°C are added. The mix is then plated onto  
prewarmed LB plates and incubated at 37°C for at least 12  
hours. From these phage, a library of 170,000 plaques in  
*E. coli* K802 is created and amplified 100-fold for future  
10 use.

To screen for the laccase gene, 25,000 plaques from the  
amplified genomic library are plated onto NZY/agarose plates  
for plaque lifts using conventional methods. Filters are  
screened using the 220 nucleotide PCR fragment randomly  
15 labelled to  $5 \times 10^8$  cpm/µg as a probe. Filters are hybridized  
in 50% formamide, 6xSSC for 16 hours at 42°C and washed with  
0.5xSSC, 0.1% SDS at 65°C. Positive clones are picked and  
rescreened using conventional methods. The nine positive  
clones identified fell into two classes that by DNA sequence  
20 analysis are shown to code for two different laccase genes,  
RSlac1 and RSlac2. The complete nucleotide sequence of each  
of these genes is determined using fluorescent nucleotides  
and an Applied Biosystems automatic DNA sequencer (Model  
363A, version 1.2.0). The nucleotide and predicted amino  
25 acid sequences are depicted in Figures 1 and 2.

For isolation of RSlac3, poly A RNA purified from *R.*  
*solani* mycelia grown in the presence of 1 mM anisidine is  
used as a template for cDNA synthesis using standard  
protocols. The cDNA is fractionated by electrophoresis  
30 through a 0.8% agarose gel and DNA fragments between 1.7 and  
3.5 kb in size are collected. A library is then created by  
cloning the size-fractionated cDNA into the yeast expression

vector pYES2. 3000 yeast transformants from this library are plated initially on YNB (1.7 g yeast nitrogen base without amino acids, 5 g  $(\text{NH}_4)_2\text{SO}_4$  per liter) with 2% glucose. After 4 days growth at 30°C, the resulting colonies are replica plated to YNB with 0.1% glucose, 2% galactose and 2mM ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid; Sigma # A-1888). After 24 hours of growth at 30°C a single colony has a light green halo which gradually turns a dark purple. The plasmid from this colony is isolated and the insert sequenced. The sequence of the translated portion of the RSlac3 gene and protein is shown in SEQ.ID NOS. 13 and 14, and in Figure 4.

3. Expression of laccase gene

The plasmid pMWR-1 is a pUC derived vector containing the TAKA amylase transcription regulation signals and the TAKA amylase signal sequence. This plasmid is engineered with a unique SfiI site at the signal sequence cleavage site, and a 3' adjacent NsiI site such that these two restriction enzymes can be used to introduce, in frame, a foreign protein. Using a PCR reaction (conducted as described above, but with 100 ng of the appropriate linearized plasmid DNA as a template) and mutagenized primers, an SfiI site is introduced at amino acid 12 and amino acid 14 of RSlac1 and RSlac2, respectively, such that the protein coding sequences are in frame with the TAKA signal sequence. In addition, a PCR amplification is also used to introduce a PstI site (CTGCAG) at the 3' end of RSlac1 and an NsiI site (ATGCAT) at the 3' end of RSlac2.

To prepare for transformation, cells of *Aspergillus oryzae* are cultivated in YPG (1g/l yeast extract, 0.25 g  $\text{K}_2\text{PO}_4$ , 0.125 g/ $\text{MgSO}_4$ , 3.75 g glucose) at 34°C with 100-120rpm

for 16-20 hours, then collected by filtration with miracloth. Cells are washed with Mg solution (0.6M  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ), then 2-6 g of cells are taken up in 10 ml  $\text{MgP}$  (1.2M  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 10mM  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ; pH 5.8). To this is  
5 added 1 ml of Novozyme® 234 (120 mg/ml  $\text{MgP}$ ), and the sample kept on ice for 5 minutes. One ml of BSA (12 mg/ml) is added, and the sample shaken gently at 34-37°C. Protoplasts are collected by filtration through miracloth, and overlain with 5 ml of ST (0.6 M Sorbitol, 100mM Tris; pH 7). The  
10 sample is spun at 2500 rpm for 15 minutes, and a band of protoplasts collected. Two volumes of STC (1.2M Sorbitol, 10 mM tris, 10 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ; pH 7.5) are added and the sample is spun at 2500 rpm for 5 minutes. The precipitate is washed twice with 5 ml of STC, and the protoplasts suspended in  
15 0.5-1ml of STC.

For the transformation process, the protoplast concentration is adjusted to  $1-5 \times 10^7/\text{ml}$ . To 100  $\mu\text{l}$  of protoplast solution is added a maximum of 10  $\mu\text{l}$  of DNA solution (5-10  $\mu\text{g}$  of supercoiled DNA) and 0.2 ml of PEG  
20 (60% PEG4000, 10mM Tris, 10mM  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ ; pH 7.5), and the combination is mixed well. The sample is kept at room temperature for 25 minutes; then to it is added first 0.2 ml PEG, with mixing, the 0.85 ml PEG with mixing. The mixture is kept at room temperature for 20 minutes, then spun at  
25 4000 rpm for 15 minutes. The precipitate is washed with 2 ml of STC by spinning at 2500 rpm for 10 minutes. The protoplasts are resuspended in 0.2-0.5 ml of STC, and then spread on COVE plates. COVE medium (pH 7) contains 342.3 g/l sucrose, 25 g/l agar and a salt solution comprising 26 g/l  
30 KCl, 26 g/l  $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ , 76 g/l  $\text{KH}_2\text{PO}_4$ , and 50 ml/l of trace metals; the trace metals are 40 mg/l  $\text{NaB}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ , 400 mg/l

CuSO<sub>4</sub>·5H<sub>2</sub>O, 1200mg/l FeSO<sub>4</sub>·7H<sub>2</sub>O, 700mg/l MnSO<sub>4</sub>·H<sub>2</sub>O, 800mg/l Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 10 g/l ZnSO<sub>4</sub>·7H<sub>2</sub>O). After autoclaving, 10 ml/l of 1M filtrated acetamide and 5-10 ml of 3M CsCl are added to the solution. Transformants are selected by growth cells  
5 on COVE medium which contains acetamide as the carbon source.

The confirmation of laccase production in the samples is determined by the ABTS oxidation method as described above on Cove medium with 2 mM ABTS, at pH 5 and 7.3. Both  
10 RSlac1 and RSlac2 express laccase activity at pH 5 and pH 7, in contrast with a control laccase which shows substantially no activity at pH 7.3.

The products of the expression of each of RSlac1 and RSlac2 are tested for oxidase activity at various pHs using  
15 syringaldazine as the substrate. The assay is conducted substantially as described above for the assay of the native protein, over pH range of 4-9. As shown in Figures 5 and 6, both laccases are active at pHs over pH 5, and RSlac1 has particularly good activity at pHs over 6. The pattern of  
20 activity is generally comparable to that observed for the RSlac3 laccase isolated from RS 22 (see Table 1 above), with RSlac1 exhibiting the broadest range of activity.

#### Deposit of Biological Materials

The following biological materials have been deposited  
25 under the terms of the Budapest Treaty in the International Mycological Institute, Genetic Resource Reference Collection, located at Bakeham Lane, Egham, Surrey TW20 9TY and given the following accession number.

30 <u>Deposit</u>	<u>Accession Number</u>
<i>Rhizoctonia solani</i> RS22	IMI CC 358730

The following biological materials have been deposited under the terms of the Budapest Treaty with the Agricultural Research Service Patent Culture Collection, Northern Regional Research Center, 1815 University Street, Peoria, Illinois, 61604 and given the following accession numbers.

<u>Deposit</u>	<u>Accession Number</u>
5 <i>E. coli</i> containing RSlac1 fused to an $\alpha$ -amylase signal sequence (EMCC 00844)	NRRL B-21141
10 <i>E. coli</i> containing RSlac2 with an SfiI site insert (EMCC 00845)	NRRL B-21142
15 <i>E. coli</i> containing RSlac3 (EMCC 0088)	NRRL B-21156

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(iii) NUMBER OF SEQUENCES: 14

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## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(A) APPLICATION NUMBER: to be assigned  
(B) FILING DATE: 13-SEP-1994

## (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/172,331  
(B) FILING DATE: 22-DEC-1993

## (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/122,230  
(B) FILING DATE: 17-SEP-1993

## (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/122,827  
(B) FILING DATE: 17-SEP-1993

## (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/162,827  
(B) FILING DATE: 03-DEC-1993

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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 2838 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Rhizoctonia laccase
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 302..351
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 463..512
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  - (B) LOCATION: 822..877
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  - (B) LOCATION: 1001..1054
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  - (B) LOCATION: 2348..2404
- (ix) FEATURE:

(A) NAME/KEY: intron  
(B) LOCATION: 2438..2498

## (ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: join(170..301, 352..462, 513..575, 634..759, 819  
..821, 878..1000, 1055..1315, 1373..1696, 1755  
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..2437, 2499..2621)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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                               Met Ala
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Arg Thr Thr Phe Leu Val Ser Val Ser Leu Phe Val Ser Ala Val Leu
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GCG CGC ACC GTC GAG TAC GGC TTG AAG ATT AGT GAT GGG GAG ATA GCT      271
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Leu Ile Phe Ala Asn Lys Gly Asp Thr Leu Lys Val Lys Val Gln Asn
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Lys Leu Thr Asn Pro Glu Met Tyr Arg Thr Thr Ser Ile
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GTAGGATTCT GGAAGGTTGG CCTGAACTCT CTGTAAACCG ACAACCCGAT GTCACCAG      633
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Cys Pro Ile Val Pro Arg Glu Ser Tyr Thr Tyr Thr Ile Pro Leu Asp
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GAT CAA ACC GGA ACC TAT TGG TAC CAT AGC CAC TTG AGT TCG CAA TAC      729
Asp Gln Thr Gly Thr Tyr Trp Tyr His Ser His Leu Ser Ser Gln Tyr
                               120                               130
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Val Asp Gly L u Arg Gly Pro Leu Val Ile
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Asp Pro His Arg Arg Leu Tyr Asp Val Asp Asp Glu Lys Thr Val Leu	
150 155 160	
ATC ATC GGT GAC TGG TAT CAT GAA TCG TCC AAG GCA ATC CTT GCT TCT	982
Ile Ile Gly Asp Trp Tyr His Glu Ser Ser Lys Ala Ile Leu Ala Ser	
165 170 175 180	
GGT AAC ATT ACC CGA CAG GTAAGTGATA CATGCCGGTC CCAGAAAAAT	1030
Gly Asn Ile Thr Arg Gln	
185	
TCTCTAAATT CATTTTAATT ACAG CGA CCG GTC TCT GCC ACC ATC AAC GGC	1081
Arg Pro Val Ser Ala Thr Ile Asn Gly	
190 195	
AAA GGT CGA TTT GAC CCT GAC AAC ACT CCT GCC AAC CCA GAT ACT CTG	1129
Lys Gly Arg Phe Asp Pro Asp Asn Thr Pro Ala Asn Pro Asp Thr Leu	
200 205 210	
TAC ACC CTC AAG GTC AAG CGA GGG AAG CGC TAT CGT CTG CGT GTC ATC	1177
Tyr Thr Leu Lys Val Lys Arg Gly Lys Arg Tyr Arg Leu Arg Val Ile	
215 220 225	
AAT AGC TCG GAG ATC GCT TCG TTC CGA TTC AGT GTG GAA GGT CAC AAG	1225
Asn Ser Ser Glu Ile Ala Ser Phe Arg Phe Ser Val Glu Gly His Lys	
230 235 240	
GTG ACT GTG ATT GCT GCC GAT GGC GTC TCT ACC AAA CCG TAT CAG GTC	1273
Val Thr Val Ile Ala Ala Asp Gly Val Ser Thr Lys Pro Tyr Gln Val	
245 250 255	
GAT GCG TTT GAT ATT CTA GCA GGA CAG CGC ATA GAT TGC GTC	1315
Asp Ala Phe Asp Ile Leu Ala Gly Gln Arg Ile Asp Cys Val	
260 265 270	
GTAAGTGTG TCCGAACCCA CATCTGAGCT CAAGTGTGTA TACATGCGCG CTTATAG	1372
GTG GAG GCG AAC CAA GAA CCC GAC ACA TAC TGG ATC AAC GCA CCG CTG	1420
Val Glu Ala Asn Gln Glu Pro Asp Thr Tyr Trp Ile Asn Ala Pro Leu	
275 280 285	
ACC AAC GTG CCC AAC AAG ACC GCT CAG GCT CTC CTC GTT TAT GAG GAG	1468
Thr Asn Val Pro Asn Lys Thr Ala Gln Ala Leu Leu Val Tyr Glu Glu	
290 295 300 305	
GAT CGT CGG CCG TAC CAC CCT CCA AAG GGC CCG TAT CGC AAG TGG AGC	1516
Asp Arg Arg Pro Tyr His Pro Pro Lys Gly Pro Tyr Arg Lys Trp Ser	
310 315 320	
GTC TCT GAG GCG ATC ATC AAG TAC TGG AAT CAC AAG CAC AAG CAC GGA	1564
Val Ser Glu Ala Ile Ile Lys Tyr Trp Asn His Lys His Lys His Gly	
325 330 335	
CGT GGT TTG CTG TCT GGA CAT GGA GGT CTC AAG GCT CGG ATG ATC GAG	1612
Arg Gly Leu Leu Ser Gly His Gly Gly Leu Lys Ala Arg Met Ile Glu	
340 345 350	
GGT AGC CAT CAT CTG CAT TCG CGC AGC GTC GTT AAG CGC CAG AAT GAG	1660

Gly	Ser	His	His	Leu	His	Ser	Arg	Ser	Val	Val	Lys	Arg	Gln	Asn	Glu		
355						360					365						
ACC	ACC	ACT	GTT	GTA	ATG	GAC	GAG	AGC	AAG	CTC	GTT	GTAAGTACCA				1706	
Thr	Thr	Thr	Val	Val	Met	Asp	Glu	Ser	Lys	Leu	Val						
370					375					380							
TATTTAAAAG	TTGGTTGGGT	TTCGAATACT	TATTTCAACT	TTTCTTAG	CCA	CTG	GAA									1763	
							Pro	Leu	Glu								
TAC	CCC	GGC	GCT	GCA	TGC	GGG	TCT	AAA	CCT	GCT	GAC	CTC	GTC	TTG	GAT		1811
Tyr	Pro	Gly	Ala	Ala	Cys	Gly	Ser	Lys	Pro	Ala	Asp	Leu	Val	Leu	Asp		
385					390					395					400		
CTC	ACT	TTT	GGT	TTG	GTATGTAGCC	AAATCGCCCA	TATACAGGAT	ACTGAATATT									1866
Leu	Thr	Phe	Gly	Leu													
				405													
GTTTGTGCGT	GTAG	AAC	TTT	GCT	ACC	GGG	CAC	TGG	ATG	ATC	AAC	GGT	ATC				1916
		Asn	Phe	Ala	Thr	Gly	His	Trp	Met	Ile	Asn	Gly	Ile				
						410						415					
CCA	TAC	GAG	TCT	CCC	AAA	ATC	CCC	ACA	TTG	CTC	AAG	ATC	CTC	ACT	GAT		1964
Pro	Tyr	Glu	Ser	Pro	Lys	Ile	Pro	Thr	Leu	Leu	Lys	Ile	Leu	Thr	Asp		
		420					425					430					
GAG	GAC	GGG	GTT	ACC	GAG	TCT	GAC	TTC	GTATGTTCCC	TTTTCGGTAT							2011
Glu	Asp	Gly	Val	Thr	Glu	Ser	Asp	Phe									
	435					440											
CTTCGTATGC	GTGCACTGAC	TCGTGCTGGT	GGGAATTTAG	ACC	AAG	GAG	GAG	CAC									2066
					Thr	Lys	Glu	Glu	His								
							445										
ACA	GTC	ATA	CTC	CCG	AAG	AAC	AAA	TGC	ATC	GAA	TTC	AAC	ATC	AAG	GGG		2114
Thr	Val	Ile	Leu	Pro	Lys	Asn	Lys	Cys	Ile	Glu	Phe	Asn	Ile	Lys	Gly		
		450					455						460				
AAC	TCG	GGT	ATT	CCC	ATT	ACG	CAC	CCC	GTA	CAT	CTT	CAC	GGT				2156
Asn	Ser	Gly	Ile	Pro	Ile	Thr	His	Pro	Val	His	Leu	His	Gly				
	465					470					475						
GTAAGTGCAT	ATCGGATGGT	TTACGATACT	AAGGCTCATC	AAC	TTTTTTAG	CAC	ACT										2212
						His	Thr										
TGG	GAT	GTC	GTA	CAA	TTT	GGC	AAC	AAC	CCA	CCC	AAT	TAT	GTC	AAT	CCT		2260
Trp	Asp	Val	Val	Gln	Phe	Gly	Asn	Asn	Pro	Pro	Asn	Tyr	Val	Asn	Pro		
480					485					490					495		
CCC	CGT	AGG	GAC	GTG	GTT	GGC	TCT	ACA	GAT	GCG	GGT	GTG	AGG	ATT	CAG		2308
Pro	Arg	Arg	Asp	Val	Val	Gly	Ser	Thr	Asp	Ala	Gly	Val	Arg	Ile	Gln		
				500					505					510			
TTC	AAG	ACC	GAC	AAT	CCA	GGA	CCG	TGG	TTC	CTG	CAC	TGC	GTGCGTCGGT				2357
Phe	Lys	Thr	Asp	Asn	Pro	Gly	Pro	Trp	Phe	Leu	His	Cys					
			515				520										
CCCCATCGTC	CGTTATGGTT	TTTCTAATAC	GTCCCCATTCT	ATTTTAG	CAT	ATT	GAC										2413
					His	Ile	Asp										
							525										
TGG	CAT	CTT	GAG	GAG	GGT	TTC	GCA	GTGAGTACTG	AGACCTAAGT	GCTACTCGGC							2467
Trp	His	Leu	Glu	Glu	Gly	Phe	Ala										
		530					535										

TCATTACTGA TTACCGCATG TATGCGTCTA G ATG GTG TTT GCT GAA GCG CCC 2519  
Met Val Phe Ala Glu Ala Pro  
540

GAA GCC GTC AAG GGA GGT CCA AAG AGC GTG GCC GTG GAC TCT CAG TGG 2567  
Glu Ala Val Lys Gly Gly Pro Lys Ser Val Ala Val Asp Ser Gln Trp  
545 550 555

GAA GGG CTG TGT GGC AAG TAC GAC AAC TGG CTA AAA TCA AAT CCG GGC 2615  
Glu Gly Leu Cys Gly Lys Tyr Asp Asn Trp Leu Lys Ser Asn Pro Gly  
560 565 570

CAG CTG TAGGCGTATC GCAGCCACAT TGGTGATGAT TGAAAGTTGC ATCTTGTTCC 2671  
Gln Leu  
575

TATAACCGGC TCTTATATAC GGGTGTCTCC CAGTAAAGTC GTAGCCCAAT TTCAGCCGAG 2731

ACAGATATTT AGTGGACTCT TACTCTTG TG TCCCATTGAC GCACATCGTT GCATCAAACC 2791

TGCTTTTAT CGTCCCTCTT TGTAATTTGT GTTGCTGTAA TGTATCG 2838

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 576 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Arg Thr Thr Phe Leu Val Ser Val Ser Leu Phe Val Ser Ala  
1 5 10 15

Val Leu Ala Arg Thr Val Glu Tyr Gly Leu Lys Ile Ser Asp Gly Glu  
20 25 30

Ile Ala Pro Asp Gly Val Lys Arg Asn Ala Thr Leu Val Asn Gly Gly  
35 40 45

Tyr Pro Gly Pro Leu Ile Phe Ala Asn Lys Gly Asp Thr Leu Lys Val  
50 55 60

Lys Val Gln Asn Lys Leu Thr Asn Pro Glu Met Tyr Arg Thr Thr Ser  
65 70 75 80

Ile His Trp His Gly Leu Leu Gln His Arg Asn Ala Asp Asp Asp Gly  
85 90 95

Pro Ser Phe Val Thr Gln Cys Pro Ile Val Pro Arg Glu Ser Tyr Thr  
100 105 110

Tyr Thr Ile Pro Leu Asp Asp Gln Thr Gly Thr Tyr Trp Tyr His Ser  
115 120 125

His Leu Ser Ser Gln Tyr Val Asp Gly Leu Arg Gly Pro Leu Val Ile  
130 135 140

Tyr Asp Pro Lys Asp Pro His Arg Arg Leu Tyr Asp Val Asp Asp Glu  
145 150 155 160

Lys Thr Val Leu Il Ile Gly Asp Trp Tyr His Glu Ser Ser Lys Ala  
165 170 175

Ile Leu Ala Ser Gly Asn Ile Thr Arg Gln Arg Pro Val Ser Ala Thr  
 180 185 190  
 Il Asn Gly Lys Gly Arg Phe Asp Pro Asp Asn Thr Pro Ala Asn Pro  
 195 200 205  
 Asp Thr Leu Tyr Thr Leu Lys Val Lys Arg Gly Lys Arg Tyr Arg Leu  
 210 215 220  
 Arg Val Ile Asn Ser Ser Glu Ile Ala Ser Phe Arg Phe Ser Val Glu  
 225 230 235 240  
 Gly His Lys Val Thr Val Ile Ala Ala Asp Gly Val Ser Thr Lys Pro  
 245 250 255  
 Tyr Gln Val Asp Ala Phe Asp Ile Leu Ala Gly Gln Arg Ile Asp Cys  
 260 265 270  
 Val Val Glu Ala Asn Gln Glu Pro Asp Thr Tyr Trp Ile Asn Ala Pro  
 275 280 285  
 Leu Thr Asn Val Pro Asn Lys Thr Ala Gln Ala Leu Leu Val Tyr Glu  
 290 295 300  
 Glu Asp Arg Arg Pro Tyr His Pro Pro Lys Gly Pro Tyr Arg Lys Trp  
 305 310 315 320  
 Ser Val Ser Glu Ala Ile Ile Lys Tyr Trp Asn His Lys His Lys His  
 325 330 335  
 Gly Arg Gly Leu Leu Ser Gly His Gly Gly Leu Lys Ala Arg Met Ile  
 340 345 350  
 Glu Gly Ser His His Leu His Ser Arg Ser Val Val Lys Arg Gln Asn  
 355 360 365  
 Glu Thr Thr Thr Val Val Met Asp Glu Ser Lys Leu Val Pro Leu Glu  
 370 375 380  
 Tyr Pro Gly Ala Ala Cys Gly Ser Lys Pro Ala Asp Leu Val Leu Asp  
 385 390 395 400  
 Leu Thr Phe Gly Leu Asn Phe Ala Thr Gly His Trp Met Ile Asn Gly  
 405 410 415  
 Ile Pro Tyr Glu Ser Pro Lys Ile Pro Thr Leu Leu Lys Ile Leu Thr  
 420 425 430  
 Asp Glu Asp Gly Val Thr Glu Ser Asp Phe Thr Lys Glu Glu His Thr  
 435 440 445  
 Val Ile Leu Pro Lys Asn Lys Cys Ile Glu Phe Asn Ile Lys Gly Asn  
 450 455 460  
 Ser Gly Ile Pro Ile Thr His Pro Val His Leu His Gly His Thr Trp  
 465 470 475 480  
 Asp Val Val Gln Phe Gly Asn Asn Pro Pro Asn Tyr Val Asn Pro Pro  
 485 490 495  
 Arg Arg Asp Val Val Gly Ser Thr Asp Ala Gly Val Arg Ile Gln Phe  
 500 505 510  
 Lys Thr Asp Asn Pro Gly Pro Trp Phe Leu His Cys His Ile Asp Trp  
 515 520 525  
 His Leu Glu Glu Gly Phe Ala Met Val Phe Ala Glu Ala Pro Glu Ala

530	535	540
Val Lys Gly Gly Pro Lys Ser Val Ala Val Asp Ser Gln Trp Glu Gly		
545	550	555 560
Leu Cys Gly Lys Tyr Asp Asn Trp Leu Lys Ser Asn Pro Gly Gln Leu		
	565	570 575

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 3117 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Rhizoctonia laccase
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: join(393..524, 577..687, 737..799, 860..985, 1043..1045, 1097..1219, 1269..1538, 1601..1996, 2047..2118, 2174..2284, 2338..2439, 2495..2635, 2693..2725, 2786..2899)
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 525..576
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 688..736
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 800..859
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 986..1042
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 1220..1268
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 1539..1600
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 1823..1936
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 1973..2046
- (ix) FEATURE:
  - (A) NAME/KEY: intron
  - (B) LOCATION: 2119..2173
- (ix) FEATURE:
  - (A) NAME/KEY: intron

(B) LOCATION: 2285..2337

## (ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 2440..2494

## (ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 2636..2692

## (ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 1046..1096

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAGTGATCCG CCAGAGTTCA GCGCGATAAG TTCCTAAATA GTCATTCGCC TATTCGTGTA	60
CCTCAGCATA CTGACGACAT ACCGCCAGAT CGCCCTCGGT TCGGGCGTGG CATACGTTCG	120
CAAGGGCACC TCACGGAGCA AACTCTAAAA AGCTTCGGCA TGGATTGCAT TTTGTATTGT	180
AAACAAGTTA CGAGAAAAAC AATAGATCAG TTTTGTCCGA ATCGGATGGC TTGAAACGGA	240
AGTACCGATG GCCGATCCGA GTCGAATGAA TTAACGCATC TGAAACGGGA CCCTGAGTCG	300
AGGCACCCGC CGGCCTTGGC CGTATAAGTC ACTTGTCGCC AACTAGCACT TTTTCATTCC	360
CCCTTTTCTT CTTCCTCGTC TTCTTCTTCT CT ATG GCT CGG TCG ACT ACT TCA	413
Met Ala Arg Ser Thr Thr Ser	
1 5	
CTC TTT GCA CTG TCT CTC GTT GCT TCA GCG TTT GCT CGA GTC GTT GAC	461
Leu Phe Ala Leu Ser Leu Val Ala Ser Ala Phe Ala Arg Val Val Asp	
10 15 20	
TAT GGG TTT GAT GTG GCT AAT GGG GCA GTT GCT CCG GAT GGT GTA ACA	509
Tyr Gly Phe Asp Val Ala Asn Gly Ala Val Ala Pro Asp Gly Val Thr	
25 30 35	
AGG AAC GCG GTT CTC GTGAGTTAGC TGTAAGATGG TGTATATGCT GGTTCCTAA	564
Arg Asn Ala Val Leu	
40	
CGGGAATGTC AG GTC AAT GGT CGC TTC CCT GGT CCA TTG ATC ACC GCC	612
Val Asn Gly Arg Phe Pro Gly Pro Leu Ile Thr Ala	
45 50 55	
AAC AAG GGG GAT ACA CTT AAA ATC ACC GTG CGG AAT AAA CTC TCC GAT	660
Asn Lys Gly Asp Thr Leu Lys Ile Thr Val Arg Asn Lys Leu Ser Asp	
60 65 70	
CCA ACT ATG CGA AGG AGC ACG ACC ATC GTTAGTACTT CCCCTCATCT	707
Pro Thr Met Arg Arg Ser Thr Thr Ile	
75 80	
GTCTTGAAAC TTTCTCATCT TTTTGAAG CAC TGG CAC GGT CTG CTC CAA CAC	760
His Trp His Gly Leu Leu Gln His	
85	
AGG ACG GCA GAA GAA GAT GGC CCG GCC TTT GTA ACC CAG GTATGCCTTA	809
Arg Thr Ala Glu Glu Asp Gly Pro Ala Phe Val Thr Gln	
90 95 100	
TCCTATCGCT GCTCTGTCCC CGCGTCCTTC CCTGACTCGG GCGATTCTAG TGC CCG	865
Cys Pro	

ATT CCT CCG CAA GAA TCG TAC ACC TAT ACG ATG CCG CTC GGC GAA CAG Ile Pro Pro Gln Glu Ser Tyr Thr Tyr Thr Met Pro Leu Gly Glu Gln 105 110 115 120	913
ACC GGC ACG TAT TGG TAC CAC AGC CAC TTG AGC TCC CAG TAT GTG GAC Thr Gly Thr Tyr Trp Tyr His Ser His Leu Ser Ser Gln Tyr Val Asp 125 130 135	961
GGG TTG CGT GGG CCC ATC GTT ATT GTAAGTCTTC ATTTAACCTT ATTCTTGTT Gly Leu Arg Gly Pro Ile Val Ile 140	1015
ATGGCTGATT GTGACGTCGT GGTTAGT ATG TTCGTGGCTT CCACAAGAAG Met 145	1065
TCAGCAGCCC TTGAAGCTAA CTTTATTCCA G GAC CCC CAC GAC CCG TAC AGA Asp Pro His Asp Pro Tyr Arg 150	1117
AAC TAC TAT GAT GTC GAC GAC GAG CGT ACG GTC TTT ACT TTA GCA GAC Asn Tyr Tyr Asp Val Asp Asp Glu Arg Thr Val Phe Thr Leu Ala Asp 155 160 165	1165
TGG TAC CAC ACG CCG TCG GAG GCT ATC ATT GCC ACC CAC GAT GTC TTG Trp Tyr His Thr Pro Ser Tyr Ala Ile Ile Ala Thr His Asp Val Leu 170 175 180	1213
AAA ACG GTACGCGTTA ATCCTTCTAG CTTTCTTTCC TTGGGTCAC TTTCTATCAG Lys Thr 185	1268
ATC CCC GAC TCG GGT ACG ATC AAC GGC AAA GGC AAA TAC GAT CCT GCT Ile Pro Asp Ser Gly Thr Ile Asn Gly Lys Gly Lys Tyr Asp Pro Ala 190 195 200	1316
TCG GCT AAC ACC AAC AAC ACG ACA CTC GAG AAC CTC TAC ACT CTC AAA Ser Ala Asn Thr Asn Asn Thr Thr Leu Glu Asn Leu Tyr Thr Leu Lys 205 210 215	1364
GTC AAA CGC GGC AAG CGG TAT CGC CTG AGG ATT ATC AAC GCC TCG GCC Val Lys Arg Gly Lys Arg Tyr Arg Leu Arg Ile Ile Asn Ala Ser Ala 220 225 230	1412
ATC GCT TCG TTC CGG TTC GGC GTG CAG GGC CAC AAG TGC ACG ATC ATC Ile Ala Ser Phe Arg Phe Gly Val Gln Gly His Lys Cys Thr Ile Ile 235 240 245 250	1460
GAG GCT GAT GGC GTC CTC ACC AAA CCG ATC GAG GTC GAT GCG TTT GAT Glu Ala Asp Gly Val Leu Thr Lys Pro Ile Glu Val Asp Ala Phe Asp 255 260 265	1508
ATT CTA GCA GGC CAG AGG TAT AGC TGC ATC GTAAGTCTAC CTATGCCTTG Ile Leu Ala Gly Gln Arg Tyr Ser Cys Ile 270 275	1558
TTGTGGAGAT AAGAACCTGA CTGAATGTAT GCGCTCCAAT AG TTG AAG GCC GAC Leu Lys Ala Asp 280	1612
CAA GAT CCT GAT TCC TAC TGG ATA AAT GCG CCA ATC ACA AAC GTT CTC Gln Asp Pro Asp Ser Tyr Trp Ile Asn Ala Pro Ile Thr Asn Val Leu 285 290 295	1660
AAC ACC AAC GTC CAG GCA TTG CTA GTG TAT GAA GAT GAC AAG CGT CCT	1708

Asn	Thr	Asn	Val	Gln	Ala	Leu	Leu	Val	Tyr	Glu	Asp	Asp	Lys	Arg	Pro	
			300					305					310			
ACT	CAC	TAC	CCC	TGG	AAG	CCG	TTT	TTG	ACA	TGG	AAG	ATA	TCA	AAT	GAA	1756
Thr	His	Tyr	Pro	Trp	Lys	Pro	Phe	Leu	Thr	Trp	Lys	Ile	Ser	Asn	Glu	
		315					320					325				
ATC	ATT	CAG	TAC	TGG	CAG	CAC	AAG	CAC	GGG	TCG	CAC	GGT	CAC	AAG	GGA	1804
Ile	Ile	Gln	Tyr	Trp	Gln	His	Lys	His	Gly	Ser	His	Gly	His	Lys	Gly	
		330				335					340					
AAG	GGG	CAT	CAT	CAT	AAA	GTC	CGG	GCC	ATT	GGA	GGT	GTA	TCC	GGG	TTG	1852
Lys	Gly	His	His	His	Lys	Val	Arg	Ala	Ile	Gly	Gly	Val	Ser	Gly	Leu	
345					350					355					360	
AGC	TCC	AGG	GTT	AAG	AGC	CGG	GCG	AGT	GAC	CTA	TCG	AAG	AAG	GCT	GTC	1900
Ser	Ser	Arg	Val	Lys	Ser	Arg	Ala	Ser	Asp	Leu	Ser	Lys	Lys	Ala	Val	
				365					370					375		
GAG	TTG	GCT	GCT	GCA	CTC	GTT	GCG	GGT	GAG	GCC	GAG	TTG	GAC	AAG	AGG	1948
Glu	Leu	Ala	Ala	Ala	Leu	Val	Ala	Gly	Glu	Ala	Glu	Leu	Asp	Lys	Arg	
			380					385					390			
CAG	AAT	GAG	GAT	AAT	TCG	ACT	ATT	GTA	TTG	GAT	GAG	ACC	AAG	CTT	ATT	1996
Gln	Asn	Glu	Asp	Asn	Ser	Thr	Ile	Val	Leu	Asp	Glu	Thr	Lys	Leu	Ile	
		395					400					405				
GTAAGTCCCT	TAATTTTTTT	CGGTGTCACG	GAAGCTAACC	CGCGTAATAG	CCG	TTG										2052
						Pro	Leu								410	
GTT	CAA	CCT	GGT	GCA	CCG	GGC	GGC	TCC	AGA	CCA	GCT	GAC	GTC	GTG	GTC	2100
Val	Gln	Pro	Gly	Ala	Pro	Gly	Gly	Ser	Arg	Pro	Ala	Asp	Val	Val	Val	
				415					420					425		
CCT	CTG	GAC	TTT	GGC	CTC	GTATGTGGCT	TCTTGTTATT	CGTCCGGAAT								2148
Pro	Leu	Asp	Phe	Gly	Leu											
				430												
GCAAACTGAT	TTGGGTGGGC	TATAG	AAC	TTT	GCC	AAC	GGA	CTG	TGG	ACG	ATA					2200
					Asn	Phe	Ala	Asn	Gly	Leu	Trp	Thr	Ile			
							435				440					
AAC	AAT	GTC	TCC	TAC	TCC	CCT	CCG	GAT	GTC	CCT	ACT	CTC	CTC	AAG	ATC	2248
Asn	Asn	Val	Ser	Tyr	Ser	Pro	Pro	Asp	Val	Pro	Thr	Leu	Leu	Lys	Ile	
			445					450					455			
TTG	ACC	GAC	AAA	GAC	AAA	GTC	GAC	GCT	TCT	GAC	TTC	GTAGGTTTCCT				2294
Leu	Thr	Asp	Lys	Asp	Lys	Val	Asp	Ala	Ser	Asp	Phe					
		460					465									
CTTCTTCTTT	TCAAAC TAGC	TACTGACATT	AAGTGAACGT	CAG	ACG	GCC	GAT	GAA								2349
					Thr	Ala	Asp	Glu								
							470									
CAC	ACG	TAT	ATT	CTT	CCA	AAG	AAC	CAA	GTT	GTC	GAG	TTG	CAC	ATC	AAG	2397
His	Thr	Tyr	Ile	Leu	Pro	Lys	Asn	Gln	Val	Val	Glu	Leu	His	Ile	Lys	
		475				480					485					
GGA	CAG	GCT	TTG	GGA	ATC	GTA	CAC	CCC	CTT	CAT	CTG	CAT	GGC			2439
Gly	Gln	Ala	Leu	Gly	Ile	Val	His	Pro	Leu	His	Leu	His	Gly			
490					495				500							
GTACGTCTTT	CTCACACTGT	TCCAGCTCCT	ATTCTCTAAC	ACACTCCTGC	GATAG	CAT										2497
						His										



GCG TTC GAC GTC GTC CAA TTC GGC GAC AAC GCT CCA AAC TAC GTG AAC Ala Phe Asp Val Val Gln Phe Gly Asp Asn Ala Pro Asn Tyr Val Asn 505 510 515 520	2545
CCT CCG CGT AGG GAT GTA GTA GGC GTA ACT GAT GCT GGA GTC CGT ATC Pro Pro Arg Arg Asp Val Val Gly Val Thr Asp Ala Gly Val Arg Ile 525 530 535	2593
CAG TTC AGA ACC GAT AAC CCG GGC CCT TGG TTC CTC CAT TGC Gln Phe Arg Thr Asp Asn Pro Gly Pro Trp Phe Leu His Cys 540 545 550	2635
GTATGCTCTT CATCTCCAC CGCTTGTTCT TTA CT TATGG TTTACCTTC GATTTAG	2692
CAC ATT GAT TGG CAC TTG GAA GAA GGA TTT GCT GTAAGTTATT ATTCCTATTC His Ile Asp Trp His Leu Glu Glu Gly Phe Ala 555 560	2745
CGAAGCATCG GGGAGATGCT AACCAAGGGT GTGTTTTAAG ATG GTA TTC GCC GAA Met Val Phe Ala Glu 565	2800
GCG CCT GAA GAT ATC AAG AAA GGC TCT CAG AGT GTC AAG CCT GAC GGA Ala Pro Glu Asp Ile Lys Lys Gly Ser Gln Ser Val Lys Pro Asp Gly 570 575 580	2848
CAA TGG AAG AAA CTA TGC GAG AAG TAT GAG AAG TTG CCT GAA GCA CTG Gln Trp Lys Lys Leu Cys Glu Lys Tyr Glu Lys Leu Pro Glu Ala Leu 585 590 595	2896
CAG TGAAGTTGCA GTTGTTCCTTCC ATTCGGGAAC TGGCTCACTA TTCCTTTTGC Gln	2949
ATAATTCGGA CTTTTATTTT GGGACATTAT TGGACTATGG ACTTGTTTGT CACACCCTCG	3009
CTCACTGTGT CCCTCGTTGA GTACCTATAC TCTATTCGTA TAGTGGGAAT ATGGAATATC	3069
GGATGTAATA AATGCTCGTG CGTTTGGTGC TCGAAATGGG GTAGGACT	3117

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 599 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Ala Arg Ser Thr Thr Ser Leu Phe Ala Leu Ser Leu Val Ala Ser 1 5 10 15
Ala Phe Ala Arg Val Val Asp Tyr Gly Phe Asp Val Ala Asn Gly Ala 20 25 30
Val Ala Pro Asp Gly Val Thr Arg Asn Ala Val Leu Val Asn Gly Arg 35 40 45
Phe Pro Gly Pro Leu Ile Thr Ala Asn Lys Gly Asp Thr Leu Lys Ile 50 55 60
Thr Val Arg Asn Lys Leu Ser Asp Pro Thr Met Arg Arg Ser Thr Thr 65 70 75 80

Ile His Trp His Gly Leu Leu Gln His Arg Thr Ala Glu Glu Asp Gly  
                             85                            90                            95  
 Pro Ala Phe Val Thr Gln Cys Pro Ile Pro Pro Gln Glu Ser Tyr Thr  
                             100                            105                            110  
 Tyr Thr Met Pro Leu Gly Glu Gln Thr Gly Thr Tyr Trp Tyr His Ser  
                             115                            120                            125  
 His Leu Ser Ser Gln Tyr Val Asp Gly Leu Arg Gly Pro Ile Val Ile  
                             130                            135                            140  
 Met Asp Pro His Asp Pro Tyr Arg Asn Tyr Tyr Asp Val Asp Asp Glu  
                             145                            150                            155                            160  
 Arg Thr Val Phe Thr Leu Ala Asp Trp Tyr His Thr Pro Ser Glu Ala  
                             165                            170                            175  
 Ile Ile Ala Thr His Asp Val Leu Lys Thr Ile Pro Asp Ser Gly Thr  
                             180                            185                            190  
 Ile Asn Gly Lys Gly Lys Tyr Asp Pro Ala Ser Ala Asn Thr Asn Asn  
                             195                            200                            205  
 Thr Thr Leu Glu Asn Leu Tyr Thr Leu Lys Val Lys Arg Gly Lys Arg  
                             210                            215                            220  
 Tyr Arg Leu Arg Ile Ile Asn Ala Ser Ala Ile Ala Ser Phe Arg Phe  
                             225                            230                            235                            240  
 Gly Val Gln Gly His Lys Cys Thr Ile Ile Glu Ala Asp Gly Val Leu  
                             245                            250                            255  
 Thr Lys Pro Ile Glu Val Asp Ala Phe Asp Ile Leu Ala Gly Gln Arg  
                             260                            265                            270  
 Tyr Ser Cys Ile Leu Lys Ala Asp Gln Asp Pro Asp Ser Tyr Trp Ile  
                             275                            280                            285  
 Asn Ala Pro Ile Thr Asn Val Leu Asn Thr Asn Val Gln Ala Leu Leu  
                             290                            295                            300  
 Val Tyr Glu Asp Asp Lys Arg Pro Thr His Tyr Pro Trp Lys Pro Phe  
                             305                            310                            315                            320  
 Leu Thr Trp Lys Ile Ser Asn Glu Ile Ile Gln Tyr Trp Gln His Lys  
                             325                            330                            335  
 His Gly Ser His Gly His Lys Gly Lys Gly His His His Lys Val Arg  
                             340                            345                            350  
 Ala Ile Gly Gly Val Ser Gly Leu Ser Ser Arg Val Lys Ser Arg Ala  
                             355                            360                            365  
 Ser Asp Leu Ser Lys Lys Ala Val Glu Leu Ala Ala Ala Leu Val Ala  
                             370                            375                            380  
 Gly Glu Ala Glu Leu Asp Lys Arg Gln Asn Glu Asp Asn Ser Thr Ile  
                             385                            390                            395                            400  
 Val Leu Asp Glu Thr Lys Leu Ile Pro Leu Val Gln Pro Gly Ala Pro  
                             405                            410                            415  
 Gly Gly Ser Arg Pro Ala Asp Val Val Val Pro Leu Asp Phe Gly Leu  
                             420                            425                            430  
 Asn Phe Ala Asn Gly Leu Trp Thr Ile Asn Asn Val Ser Tyr Ser Pro

	435						440				445					
Pro	Asp 450	Val	Pro	Thr	Leu	Leu 455	Lys	Ile	Leu	Thr	Asp 460	Lys	Asp	Lys	Val	
Asp 465	Ala	Ser	Asp	Phe	Thr 470	Ala	Asp	Glu	His	Thr 475	Tyr	Ile	Leu	Pro	Lys 480	
Asn	Gln	Val	Val	Glu 485	Leu	His	Ile	Lys	Gly 490	Gln	Ala	Leu	Gly	Ile 495	Val	
His	Pro	Leu	His 500	Leu	His	Gly	His	Ala 505	Phe	Asp	Val	Val	Gln 510	Phe	Gly	
Asp	Asn	Ala 515	Pro	Asn	Tyr	Val	Asn 520	Pro	Pro	Arg	Arg	Asp 525	Val	Val	Gly	
Val	Thr 530	Asp	Ala	Gly	Val	Arg 535	Ile	Gln	Phe	Arg	Thr 540	Asp	Asn	Pro	Gly	
Pro 545	Trp	Phe	Leu	His	Cys 550	His	Ile	Asp	Trp	His 555	Leu	Glu	Glu	Gly	Phe 560	
Ala	Met	Val	Phe	Ala 565	Glu	Ala	Pro	Glu	Asp 570	Ile	Lys	Lys	Gly	Ser 575	Gln	
Ser	Val	Lys	Pro 580	Asp	Gly	Gln	Trp	Lys 585	Lys	Leu	Cys	Glu	Lys 590	Tyr	Glu	
Lys	Leu	Pro 595	Glu	Ala	Leu	Gln										

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ala Val Arg Asn Tyr Lys Phe Asp Ile Lys Asn Val Asn Val Ala Pro  
1 5 10 15

Asp Gly Phe Gln Arg Pro Ile Val Ser Val  
20 25

(2) INFORMATION FOR SEO ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 20 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ser Gln Tyr Val Asp Gly Leu Arg Gly Pro Leu Val Ile Tyr Asp Pro  
1 5 10 15  
Asp Asp Asp His  
20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 amino acids

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ser Arg Tyr Asx Val Asx Asx Ala Ser Thr Val Val Met Leu Glu Asx  
1 5 10 15

Trp Tyr Arg Thr Pro Ala Xaa Val Leu Glu  
20 25

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ser Leu Gly Pro Thr Pro Asn Tyr Val Asn Pro Xaa Ile Arg Asp Val  
1 5 10 15

Val Arg Val Gly Gly Thr Thr Val Val  
20 25

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gly Leu Ala Leu Val Phe Ala Glu Ala Pro Ser Gln Ile Arg Gln Gly  
1 5 10 15

Val Gln Ser Val Gln Pro Asp Asp Ala  
20 25

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 14 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ile Arg Tyr Val Gly Gly Pro Ala Val Xaa Arg Ser Val Ile  
1                      5                      10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ile Leu Ala Asn Pro Ala  
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 8 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Tyr Glu Ala Pro Ser Leu Pro Thr  
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1912 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Rhizoctonia laccase

(ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 85..1671

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTAACGCTTG GTGCCGAGCT CGGATCCACT AGTAACGCGC GCCAGTGTGC TGAATTCGC	60
GGCCGCGTCG ACACCTCCTT CAAG ATG CTT TCT AGC ATT ACC CTC CTA CCT	111
Met Leu Ser Ser Ile Thr Leu Leu Pro	
1 5	
TTG CTC GCT GCG GTC TCA ACC CCC GCC TTT GCT GCC GTC CGC AAC TAT	159
Leu Leu Ala Ala Val Ser Thr Pro Ala Phe Ala Ala Val Arg Asn Tyr	
10 15 20 25	
AAG TTC GAC ATC AAG AAC GTC AAT GTC GCT CCC GAT GGC TTT CAG CGC	207
Lys Phe Asp Ile Lys Asn Val Asn Val Ala Pro Asp Gly Phe Gln Arg	
30 35 40	
TCT ATC GTC TCC GTC AAC GGT TTA GTT CCT GGC ACG TTG ATC ACG GCC	255
Ser Ile Val Ser Val Asn Gly Leu Val Pro Gly Thr Leu Ile Thr Ala	
45 50 55	
AAC AAG GGT GAC ACC TTG CGC ATT AAT GTC ACG AAT CAA CTC ACG GAC	303
Asn Lys Gly Asp Thr Leu Arg Ile Asn Val Thr Asn Gln Leu Thr Asp	
60 65 70	
CCT AGT ATG CGT CGT GCC ACA ACG ATT CAT TGG CAT GGA TTG TTC CAA	351
Pro Ser Met Arg Arg Ala Thr Thr Ile His Trp His Gly Leu Phe Gln	
75 80 85	

GCT ACT ACC GCC GAC GAG GAT GGC CCC GCA TTC GTC ACG CAA TGC CCT Ala Thr Thr Ala Asp Glu Asp Gly Pro Ala Phe Val Thr Gln Cys Pro 90 95 100 105	399
ATT GCG CAA AAT TTG TCC TAT ACA TAC GAG ATC CCA TTG CGC GGC CAA Ile Ala Gln Asn Leu Ser Tyr Thr Tyr Glu Ile Pro Leu Arg Gly Gln 110 115 120	447
ACA GGA ACC ATG TGG TAT CAC GCC CAT CTT GCG AGT CAA TAT GTC GAT Thr Gly Thr Met Trp Tyr His Ala His Leu Ala Ser Gln Tyr Val Asp 125 130 135	495
GGA TTG CGA GGC CCT TTG GTC ATC TAT GAT CCA AAC GAC CCA CAC AAG Gly Leu Arg Gly Pro Leu Val Ile Tyr Asp Pro Asn Asp Pro His Lys 140 145 150	543
TCG CGC TAC GAC GTG GAT GAT GCG AGC ACA GTA GTC ATG CTT GAG GAC Ser Arg Tyr Asp Val Asp Asp Ala Ser Thr Val Val Met Leu Glu Asp 155 160 165	591
TGG TAC CAT ACT CCG GCA CCC GTT CTA GAA AAG CAA ATG TTC TCG ACT Trp Tyr His Thr Pro Ala Pro Val Leu Glu Lys Gln Met Phe Ser Thr 170 175 180 185	639
AAT AAC ACC GCT CTG CTC TCT CCT GTT CCG GAC TCG GGT CTT ATC AAT Asn Asn Thr Ala Leu Leu Ser Pro Val Pro Asp Ser Gly Leu Ile Asn 190 195 200	687
GGC AAA GGG CGC TAT GTG GGC GGT CCC GCA GTT CCC CGG TCA GTA ATC Gly Lys Gly Arg Tyr Val Gly Gly Pro Ala Val Pro Arg Ser Val Ile 205 210 215	735
AAC GTA AAA CGT GGG AAA CGA TAT CGC TTG CGC GTA ATC AAC GCT TCT Asn Val Lys Arg Gly Lys Arg Tyr Arg Leu Arg Val Ile Asn Ala Ser 220 225 230	783
GCT ATC GGG TCG TTT ACC TTT TCG ATC GAA GGA CAT AGT CTG ACT GTC Ala Ile Gly Ser Phe Thr Phe Ser Ile Glu Gly His Ser Leu Thr Val 235 240 245	831
ATT GAG GCC GAT GGG ATC CTG CAC CAG CCC TTG GCT GTT GAC AGC TTC Ile Glu Ala Asp Gly Ile Leu His Gln Pro Leu Ala Val Asp Ser Phe 250 255 260 265	879
CAG ATT TAC GCT GGA CAA CGC TAC TCT GTC ATC GTT GAA GCC AAC CAA Gln Ile Tyr Ala Gly Gln Arg Tyr Ser Val Ile Val Glu Ala Asn Gln 270 275 280	927
ACC GCC GCC AAC TAC TGG ATT CGT GCA CCA ATG ACC GTT GCA GGA GCC Thr Ala Ala Asn Tyr Trp Ile Arg Ala Pro Met Thr Val Ala Gly Ala 285 290 295	975
GGA ACC AAT GCA AAC TTG GAC CCC ACC AAT GTC TTT GCC GTA TTG CAC Gly Thr Asn Ala Asn Leu Asp Pro Thr Asn Val Phe Ala Val Leu His 300 305 310	1023
TAC GAG GGA GCG CCC AAC GCC GAA CCC ACG ACG GAA CAA GGC AGT GCT Tyr Glu Gly Ala Pro Asn Ala Glu Pro Thr Thr Glu Gln Gly Ser Ala 315 320 325	1071
ATC GGT ACT GCA CTC GTT GAA GAG AAC CTC CAT GCG CTC ATC AAC CCT Ile Gly Thr Ala Leu Val Glu Glu Asn Leu His Ala Leu Ile Asn Pro 330 335 340 345	1119
GGC GCT CCG GGC GGC TCC GCT CCC GCA GAC GTT TCC CTC AAT CTT GCA Gly Ala Pro Gly Gly Ser Ala Pro Ala Asp Val Ser Leu Asn Leu Ala 350 355 360	1167

ATT GGG CGC AGC ACA GTT GAT GGG ATT CTT AGG TTC ACA TTT AAT AAC Ile Gly Arg Ser Thr Val Asp Gly Ile Leu Arg Phe Thr Phe Asn Asn 365 370 375	1215
ATC AAG TAC GAG GCT CCT TCG TTG CCC ACG CTC TTG AAG ATT TTG GCA Ile Lys Tyr Glu Ala Pro Ser Leu Pro Thr Leu Leu Lys Ile Leu Ala 380 385 390	1263
AAC AAT GCG AGC AAT GAC GCC GAT TTC ACG CCA AAT GAG CAC ACT ATC Asn Asn Ala Ser Asn Asp Ala Asp Phe Thr Pro Asn Glu His Thr Ile 395 400 405	1311
GTA TTG CCA CAC AAT AAA GTT ATC GAG CTC AAT ATC ACC GGA GGT GCA Val Leu Pro His Asn Lys Val Ile Glu Leu Asn Ile Thr Gly Gly Ala 410 415 420 425	1359
GAC CAC CCT ATC CAT CTC CAC GGC CAT GTG TTT GAT ATC GTC AAA TCA Asp His Pro Ile His Leu His Gly His Val Phe Asp Ile Val Lys Ser 430 435 440	1407
CTC GGT GGT ACC CCG AAC TAT GTC AAC CCG CCA CGC AGG GAC GTA GTT Leu Gly Gly Thr Pro Asn Tyr Val Asn Pro Pro Arg Arg Asp Val Val 445 450 455	1455
CGT GTC GGA GGC ACC GGT GTG GTA CTC CGA TTC AAG ACC GAT AAC CCA Arg Val Gly Gly Thr Gly Val Val Leu Arg Phe Lys Thr Asp Asn Pro 460 465 470	1503
GGC CCA TGG TTT GTT CAC TGC CAC ATT GAC TGG CAC TTG GAG GCT GGG Gly Pro Trp Phe Val His Cys His Ile Asp Trp His Leu Glu Ala Gly 475 480 485	1551
CTC GCA CTT GTC TTT GCC GAG GCC CCC AGC CAG ATT CGC CAG GGT GTC Leu Ala Leu Val Phe Ala Glu Ala Pro Ser Gln Ile Arg Gln Gly Val 490 495 500 505	1599
CAG TCG GTC CAG CCC AAC AAT GCC TGG AAC CAG CTC TGC CCC AAG TAC Gln Ser Val Gln Pro Asn Asn Ala Trp Asn Gln Leu Cys Pro Lys Tyr 510 515 520	1647
GCG GCT CTT CCT CCC GAT TTG CAG T Ala Ala Leu Pro Pro Asp Leu Gln 525	1672

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 529 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Met Leu Ser Ser Ile Thr Leu Leu Pro Leu Leu Ala Ala Val Ser Thr  
1 5 10 15

Pro Ala Phe Ala Ala Val Arg Asn Tyr Lys Phe Asp Ile Lys Asn Val  
20 25 30

Asn Val Ala Pro Asp Gly Phe Gln Arg Ser Ile Val Ser Val Asn Gly  
35 40 45

Leu Val Pro Gly Thr Leu Ile Thr Ala Asn Lys Gly Asp Thr Leu Arg  
50 55 60



Ile Asn Val Thr Asn Gln Leu Thr Asp Pro Ser Met Arg Arg Ala Thr  
 65 70 75 80  
 Thr Ile His Trp His Gly Leu Phe Gln Ala Thr Thr Ala Asp Glu Asp  
 85 90 95  
 Gly Pro Ala Phe Val Thr Gln Cys Pro Ile Ala Gln Asn Leu Ser Tyr  
 100 105 110  
 Thr Tyr Glu Ile Pro Leu Arg Gly Gln Thr Gly Thr Met Trp Tyr His  
 115 120 125  
 Ala His Leu Ala Ser Gln Tyr Val Asp Gly Leu Arg Gly Pro Leu Val  
 130 135 140  
 Ile Tyr Asp Pro Asn Asp Pro His Lys Ser Arg Tyr Asp Val Asp Asp  
 145 150 155 160  
 Ala Ser Thr Val Val Met Leu Glu Asp Trp Tyr His Thr Pro Ala Pro  
 165 170 175  
 Val Leu Glu Lys Gln Met Phe Ser Thr Asn Asn Thr Ala Leu Leu Ser  
 180 185 190  
 Pro Val Pro Asp Ser Gly Leu Ile Asn Gly Lys Gly Arg Tyr Val Gly  
 195 200 205  
 Gly Pro Ala Val Pro Arg Ser Val Ile Asn Val Lys Arg Gly Lys Arg  
 210 215 220  
 Tyr Arg Leu Arg Val Ile Asn Ala Ser Ala Ile Gly Ser Phe Thr Phe  
 225 230 235 240  
 Ser Ile Glu Gly His Ser Leu Thr Val Ile Glu Ala Asp Gly Ile Leu  
 245 250 255  
 His Gln Pro Leu Ala Val Asp Ser Phe Gln Ile Tyr Ala Gly Gln Arg  
 260 265 270  
 Tyr Ser Val Ile Val Glu Ala Asn Gln Thr Ala Ala Asn Tyr Trp Ile  
 275 280 285  
 Arg Ala Pro Met Thr Val Ala Gly Ala Gly Thr Asn Ala Asn Leu Asp  
 290 295 300  
 Pro Thr Asn Val Phe Ala Val Leu His Tyr Glu Gly Ala Pro Asn Ala  
 305 310 315 320  
 Glu Pro Thr Thr Glu Gln Gly Ser Ala Ile Gly Thr Ala Leu Val Glu  
 325 330 335  
 Glu Asn Leu His Ala Leu Ile Asn Pro Gly Ala Pro Gly Gly Ser Ala  
 340 345 350  
 Pro Ala Asp Val Ser Leu Asn Leu Ala Ile Gly Arg Ser Thr Val Asp  
 355 360 365  
 Gly Ile Leu Arg Phe Thr Phe Asn Asn Ile Lys Tyr Glu Ala Pro Ser  
 370 375 380  
 Leu Pro Thr Leu Leu Lys Ile Leu Ala Asn Asn Ala Ser Asn Asp Ala  
 385 390 395 400  
 Asp Phe Thr Pro Asn Glu His Thr Ile Val Leu Pro His Asn Lys Val  
 405 410 415  
 Ile Glu Leu Asn Ile Thr Gly Gly Ala Asp His Pro Ile His Leu His

420					425					430					
Gly	His	Val	Phe	Asp	Ile	Val	Lys	Ser	Leu	Gly	Gly	Thr	Pro	Asn	Tyr
	435						440					445			
Val	Asn	Pro	Pro	Arg	Arg	Asp	Val	Val	Arg	Val	Gly	Gly	Thr	Gly	Val
	450					455					460				
Val	Leu	Arg	Phe	Lys	Thr	Asp	Asn	Pro	Gly	Pro	Trp	Phe	Val	His	Cys
	465					470					475				480
His	Ile	Asp	Trp	His	Leu	Glu	Ala	Gly	Leu	Ala	Leu	Val	Phe	Ala	Glu
				485					490					495	
Ala	Pro	Ser	Gln	Ile	Arg	Gln	Gly	Val	Gln	Ser	Val	Gln	Pro	Asn	Asn
			500					505					510		
Ala	Trp	Asn	Gln	Leu	Cys	Pro	Lys	Tyr	Ala	Ala	Leu	Pro	Pro	Asp	Leu
		515					520					525			
Gln															

What we claim is:

1. A nucleic acid fragment containing a nucleic acid sequence encoding a *Rhizoctonia* laccase which functions optimally at  
5 pH between about 6.0 and 8.5.

2. The fragment of Claim 1 which comprises a sequence encoding a *Rhizoctonia solani* laccase.

10 3. The fragment of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 2.

4. The fragment of Claim 1 which comprises a nucleic acid  
15 sequence encoding the amino acid sequence depicted in SEQ ID NO. 4.

5. The fragment of Claim 1, which comprises a nucleic acid sequence encoding a protein containing one or more of the  
20 amino acid sequences depicted in SEQ. ID NOS. 5, 6, 7, 8, 9, 10, 11, or 12.

6. The fragment of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID  
25 NO. 14.

7. The fragment of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 1.

30 8. The fragment of Claim 1, which comprises the nucleic acid sequence depicted in SEQ. ID. NO. 3.

9. The fragment of Claim 1, which comprises the nucleic acid sequence depicted in SEQ. ID. NO. 13.
10. The fragment of Claim 1, which comprises the nucleic acid sequence contained in NRRL B-21141.
11. The fragment of Claim 1, which comprises the nucleic acid sequence contained in NRRL B-21142.
12. The fragment of Claim 1, which comprises the nucleic acid sequence encoding the laccase produced by RS 22.
13. The fragment of Claim 1, which comprises the nucleic acid sequence contained in NRRL B-21156.
14. A substantially pure *Rhizoctonia* laccase enzyme which functions optimally at a pH between about 6.0-8.5.
15. The enzyme of Claim 14 which is a *Rhizoctonia solani* laccase.
16. The enzyme of Claim 14 which comprises the sequence depicted in SEQ ID NO. 2, or a sequence with at least 80% homology thereto.
17. The enzyme of Claim 14 which comprises the sequence depicted in SEQ ID NO 4, or a sequence with at least 80% homology thereto.
18. The enzyme of Claim 14 which comprises one or more of the peptide sequences depicted in SEQ ID NOS.5, 6, 7,

8, 9, 10, 11 or 12, or a sequence with at least 80% homology to one or more of these peptides.

19. The enzyme of Claim 14 which comprises the sequence  
5 depicted in SEQ ID NO 14, or a sequence with at least 80% homology thereto.

20. A recombinant vector comprising a nucleic acid fragment containing a nucleic acid sequence encoding a *Rhizoctonia*  
10 laccase which functions optimally at pH between about 6.0-8.5.

21. The vector of Claim 20 in which the fragment is operably linked to a promoter sequence.

15

22. The vector of Claim 21 in which the promoter is a fungal or yeast promoter.

23. The vector of Claim 22 in which the promoter is the  
20 TAKA amylase promoter of *Aspergillus oryzae*.

24. The vector of Claim 22 in which the promoter is the glucoamylase (gluA) promoter of *Aspergillus niger* or *Aspergillus awamsii*.

25

25. The vector of Claim 21 which also comprises a selectable marker.

26. The vector of Claim 25 in which the selectable marker  
30 is the amdS marker of *Aspergillus nidulans* or *Aspergillus oryzae*.

27. The vector of Claim 25 in which the selectable marker is the pyrG marker of *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus awamorii*, or *Aspergillus oryzae*.
- 5 28. The vector of Claim 21 which comprises both the TAKA amylase promoter of *Aspergillus oryzae* and the amdS or pyrG marker of *Aspergillus nidulans* or *Aspergillus oryzae*.
29. A host cell comprising a heterologous nucleic acid  
10 fragment containing a nucleic acid sequence encoding a *Rhizoctonia* laccase which functions optimally at pH between about 6.0-8.5.
30. The host cell of Claim 28 which is a fungal cell.  
15
31. The host cell of Claim 30 which is an *Aspergillus* cell.
32. The host cell of Claim 29 in which the fragment is integrated into the host cell genome.  
20
33. The host cell of Claim 29 in which the fragment is contained on a vector.
34. The host cell of Claim 29 which comprises a fragment  
25 containing a sequence encoding the amino acid sequence depicted in SEQ ID NO. 2.
35. The host cell of Claim 29 which comprises a fragment containing a sequence encoding the amino acid sequence  
30 depicted in SEQ ID NO: 4.

36. The host cell of Claim 29 which comprises a fragment containing a sequence encoding the amino acid sequence depicted in SEQ ID NO: 14.
- 5 37. The host cell of Claim 29 which comprises a fragment containing a sequence encoding one or more of the amino acid sequences depicted in SEQ ID NOS.: 5, 6, 7, 8, 9, 10, 11, or 12.
- 10 38. A method for obtaining a laccase enzyme which functions optimally at a pH between about 6.0-8.5 which comprises culturing a host cell comprising a nucleic acid fragment containing a nucleic acid sequence encoding a *Rhizoctonia* laccase enzyme which functions optimally at a pH between  
15 about 6.0-8.5, under conditions conducive to expression of the enzyme, and recovering the enzyme from the culture.
39. A method for polymerizing a lignin or lignosulfate substrate in solution which comprises contacting the  
20 substrate with a *Rhizoctonia* laccase which functions optimally at a pH between about 6.0-8.5.
40. A method for in situ depolymerization in Kraft pulp which comprises contacting the pulp with a *Rhizoctonia*  
25 laccase which functions optimally at a pH between about 6.0-8.5.
41. A method for oxidizing dyes which comprises contacting the dye with a *Rhizoctonia* laccase which functions optimally  
30 at a pH between about 6.0-8.5.

42. A method of polymerizing a phenolic compounds which comprises contacting the phenolic compound with a *Rhizoctonia* laccase which functions optimally at a pH between about 6.0-8.5.

5



1 AGCGTCACACAGACATCGGATGAAACGGAAAGTGATGCGCCATTGACGCTCGCGGC 60  
 61 AACCACTGTTTCATCTCGCGAGCTAACATGGCGGACGTATAAGAGAACGCGAGAATGGGC 120  
 121 AGATTTCGATATCCCCCTCTCGTCTCGGTTTGGTCTCGGCTTGCCCTCTAATGGCGCGCAC 180  
     M A R T 4  
 181 CACTTCCCTTGTCCTCGGTTTCGCTCTTTGTTCCGCTGTCTTGCGCGCACCCGTCGAGTA 240  
     4 T F L V S V S L F V S A V L A R T V E Y 24  
 241 CGGCTTGAAGATTAGTGATGGGAGATAGCTCCTGACGGTGTTAAGCGTAATGCGACTTT 300  
     24 G L K I S D G E I A P D G V K R N A T L 44  
 301 GGgtacgcactccttgtaatccaacaattcaagtttctgatgcttggtcagTAAATGGA 360  
     1 2 1 44 V N G 47  
 361 GGGTATCCCCGGTCCACTCATTTTGGCCAACAAGGGGATACTCTCAAAGTCAAGGTCCAA 420  
     47 G Y P G P L I F A N K G D T L K V K V Q 67  
 421 AACAAAGCTACGAATCCTGAGATGTATCGCACCCACTTCCATCgtatgttcggttcgatatc 480  
     67 N K L T N P E M Y R T T S I 81  
 481 tactaatacatccgctcgctaaatatcttgtagCATTTGGCACGGTCTCTTACAACATAGAA 540  
     81 H W H G L L Q H R 90

FIG. 1A

541	ACGCCGACGACGACGGTCCTTCGTTTCGTCACCTCAGgtaggattcttgaagggttgccctga	600
90	N A D D G P S F V T Q	102
601	actctctgttaaccgacaaacccgatgtcaccagTCCCCGATGTTCCACGCGAGTCGTAT	660
102	C P I V P R E S Y	111
661	ACTTACACCATACCTCTGGACGATCAAAACCGGAACCTATTGGTACCATAGCCACTTGAGT	720
111	T Y T I P L D D Q T G T Y W Y H S H L S	131
721	TCGCAATACGTTGATGGTCTTCGAGGCCCGCTGGTAATCTgtgagtatcttgacttgctt	780
131	S Q Y V D G L R G P L V I	144
781	actgaaggcaacgagactaaaaaagcgctcgattcacagATGgttcgctctcccccttatt	840
144	Y	145
841	tagctctggatcttcatcttcacgtaatacatgatagATCCCAAGGATCCTCACAGGCG	900
144	D P K D P H R R	152
901	TTTGTAATGATGTTGACGATGAGAAGACCGTCCTGATCATCGGTGACTGGTATCATGAATC	960
152	L Y D V D D E K T V L I I G D W Y H E S	172
961	GTCCAAGGCAATCCTTCTCTGGTAACATTACCCGACAGtaagtatacatgcccgtcc	1020
172	S K A I L A S G N I T R Q	185

FIG. 1B

```

1021 cagaaaaattctctaaattcattttaaattacagGCGACGGTCTCTGCCACCATCAACGG 1080
185      R P V S A T I N G
1081 CAAAGGTCGATTGACCCCTGACAACACTCCTGCCAACCCAGATACTCTGTACACCCCTCAA 1140
194      K G R F D P D N T P A N P D T L Y T L K
1141 GGTC AAGCGAGGGAAGCGTATCGTCTGCCGTGTCAATCAATAGCTCGGAGATCGCTTCGTT 1200
214      V K R G K R Y R L R V I N S S E I A S F
1201 CCGATT CAGTGTGAAGGTCACAAGGTGACTGTGATTGCTGCCGATGGCGTCTCTACCAA 1260
234      R F S V E G H K V T V I A A D G V S T K
1261 ACCGTATCAGGTCGATGCGTTTGATATTCTAGCAGGACAGCGCATAGATTGCGTCgtaag 1320
254      P Y Q V D A F D I L A G Q R I D C V
1321 tgcgtccgaaccacatctgagctcaagtgtgatatacatgcgcgttatagGTGGAGGC 1380
272      V E A
1381 GAACCAAGAACCCGACACATACTGGATCAACGCACCGCTGACCAACGTGCCCAACAAGAC 1440
275      N Q E P D T Y W I N A P L T N V P N K T
1441 CGCTCAGGCTCTCCTCGTTTATGAGGAGGATCGTCGGCCGTACCACCCCTCCAAAGGGCCC 1500
295      A Q A L L V Y E E D R R P Y H P P K G P
1501 GTATCGCAAGTGGAGCGTCTCTGAGGCGGATCATCAAGTACTGGAATCACAGCACAAAGCA 1560
315      Y R K W S V S E A I I K Y W N H K H K H

```

FIG. 1C

```

1561 CGGACGTGTTTGTCTGGACATGGAGGTCTCAAGGCTCGGATGATCGAGGGTAGCCA 1620
335   G R G L L S G H G G L K A R M I E G S H 340

1621 TCATCTGCATTCGGCAGCGTCGTTAAGCGCCAGAATGAGACCACTGTTGTAATGGA 1680
340   H L H S R S V V K R Q N E T T V V M D 350

1681 CGAGAGCAAGCTCGTTgtaagtaaccataatttaaaagtgggttgcgaatacttatt 1740
350   E S K L V

1741 tcaacttttcttagCCACTGGAATACCCCGCGCTGCATCGGGTCTAAACCTGCTGACC 1800
350   P L E Y P G A A C G S K P A D 365

1801 TCGTCTTGGATCTCACTTTTGGTTTGGtatgtagccaaatcgcccatatacaggatactg 1860
365   L V L D L T F G L 374

1861 aatatgtttgtgcggtgtagAACTTIGCTACCGGCACTGGATGATCAACGGTATCCCAT 1920
374   N F A T G H W M I N G I P 387

1921 ACGAGTCTCCCAAAATCCCCACATTGCTCAAGATCCTCACTGATGAGGACGGGGTTACCG 1980
387   Y E S P K I P T L L K I L T D E D G V T 407

1981 AGTCTGACTTgtatgttcccttttcggtatcttcgtatgcgtgcactgcgtgctggt 2040
407   E S D F 411

2041 gggaatttagCACCAAGGAGGAGCACACAGTCATACTCCCGAAGAACAATGCATCGAAT 2100
411   T K E E H T V I L P K N K C I E 427

```

FIG. 1D

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2101 TCAACATCAAGGGAACTCGGGTATTCCATTACGCACCCCGTACATCTTCACGGTgtaa 2160  
 427 F N I K G N S G I P I T H P V H L H G 446  
  
 2161 gtgcataatcggtggtttacgataactaaggctcatcaacttttagCACACTTGGGATGT 2220  
 446 H T W D V 451  
  
 2221 CGTACAAATTGGCAACAACCCACCAATTATGTCAATCTCCCGTAGGGACGTGGTTGG 2280  
 451 V Q F G N N P P N Y V N P P R R D V V G 471  
  
 2281 CTCTACAGATCGCGGTGTGAGGATTCAAGACCGACAATCCAGGACCGTGGTTCCT 2340  
 471 S T D A G V R I Q F K T D N P G P W F L 491  
  
 2341 GCACTGgtgcgtcggtcccccatcggtcggttatgggttttctaataacgtccccattctattt 2400  
 491 H C 493  
  
 2401 tagCCATATTGACTGGCATCTTGAGGAGGTTTCGCAAgtagtactgagacctaagtgc 2460  
 493 H I D W H L E E G F A 504  
  
 2461 tactcggctcattactgattaccgcatgtatgcgtctagTGGTGTTCCTGAAGCGCCCG 2520  
 504 M V F A E A P 511  
  
 2521 AAGCCGTCAGGGAGGTCCAAAGAGCGTGGCCGTGGACTCTCAGTGGGAAGGGCTGTGTG 2580  
 511 E A V K G G P K S V A V D S Q W E G L C 531  
  
 2581 GCAAGTACGACAACTGGCTAAATCAAATCCGGCCAGCTGTAGGCGTATCGCAGCCACA 2640  
 531 G K Y D N W L K S N P G Q L \* 545

FIG. 1E

2641 TTGGTGATGATTGAAAGTTGCATCTTGTTCCCTATAACCGGCTCTTATATACGGGGTCTC 2700  
2701 CCAGTAAAGTCGTAGCCCAATTTCAGCCGAGACAGATAATTTAGTGGACTCTTACTCTTGT 2760  
2761 GTCCCATTGACGCACATCGTTGCATCAAAACCTGCTTTTATCGTCCCTCTTTGTAAATTG 2820  
2821 TGTTCGTAAATGTATCG 2838

FIG. 1F

1 AAGCTTCGGCATGGATTGCATTTTGTATTGT 180  
181 AAACAAGTTACGAGAAAAACAATAGATCAGTTTIGCCGAATCGGATGGCTTGAAACGGA 240  
241 AGTACCGATGGCCGATCCGAGTCGAATGAATTAACGCATCTGAAACGGACCCCTGAGTCG 300  
301 AGGCACCCGCGGCCCTTGGCCCGTATAAGTCACCTGTGCGCCAACTAGCACATTTTTCATTCC 360  
361 CCCTTTCTTCTTCCCTCGTCTTCTTCTCTATGGCTCGGTCGACTACTTCACCTCTTG 420  
1 M A R S T T S L F 10  
421 CACTGTCTCTGGCCGCACCGCCCTTGGCTCGAGTCGTGACTATGGGTTTGATGTGGCTA 480  
10 A L S L A A P A L A R V V D Y G F D V A 30  
481 ATGGGGCAGTTGCTCCGGATGTTGTAACAAGGAACGGGTTCTCGgtgagttagctgtaa 540  
30 N G A V A P D G V T R N A V L 45  
541 gatgggtatatgctggttgccctaacgggaatgtcagTCAATGGTCGCTCCCTGGTCCA 600  
45 V N G R F P G P 53  
601 TTGATCACCGCCAACAAGGGGATACACTTAAATCACCGTCCGGAATAAACTCTCCGAT 660  
53 L I T A N K G D T L K I T V R N K L S D 73

FIG. 2A

```

661 CCAACTATGCGAAGGAGCAGACCATCgtagtacttccccctcatctgtcttgaaacttt 720
73 P T M R R S T T I 82

721 ctcatcttttttgaagCACTGGCACGGTCTGCTCCAACACAGGACGGCAGAAGAAGATGG 780
82 H W H G L L Q H R T A E D G 97

781 CCGGGCCTTTGTAAACCCAGgtatgccttatcctatcgctgctctgtccccgcgtccttcc 840
97 P A F V T Q 103

841 ctgactcgggcgattctagTGCCCCGATTCTCCGCAAGAAATCGTACACCTATACGATGCC 900
103 C P I P P Q E S Y T Y T M P 117

901 GCTCGGCGAACAGACCGGCACGTATTGGTACCACAGCCACTTGAGCTCCCAGTATGTGGA 960
117 L G E Q T G T Y W Y H S H L S S Q Y V D 137

961 CGGGTTGCGTGCGGCCCATCGTTATTgtaagtcttcaattaaaccttattcttggctatgg 1020
137 G L R G P I V I 145

1021 ctgattgtgacgtcgtggttagATGgttcgtggtgtccacaagaagtcagcagcccttga 1080
145 Y 145

1081 agctaaactttatccagACCCCCACGACCCCGTACAGAAACTACTATGATGTCGACGACGA 1140
145 D P H D P Y R N Y Y D V D D E 160

1141 GCGTACGGTCTTTTACTTTAGCAGACTGGTACCACACGCCGTCGGAGGCTATCATTTGCCAC 1200
160 R T V F T L A D W Y H T P S E A I I A T 180

```

FIG. 2B



1201 CCACGATGTCTTGAAAAcgtacgcgttaatccttcttagctttcttcccttgggtcacttt 1260  
 180 H D V L K T 185  
 1261 ctatcagATCCCCGACTCGGGTACGATCAACGGCAAGCAATAcGATCCTGCTTCGG 1320  
 185 I P D S G T I N G K G K Y D P A S 202  
 1321 CTAACACCAACAACACGACACTCGAGAACCTCTACACTCTCAAGTCAAAACGGCGCAAGC 1380  
 202 A N T N N T T L E N L Y T L K V K R G K 222  
 1381 GGTATCGCCTGAGGATTATCAACGCCCTCGGCCATCGCTTCGTTCCGGTTCGGCGTGCAGG 1440  
 222 R Y R L R I I N A S A I A S F R F G V Q 242  
 1441 GCCACAAGTGCACGATCATCGAGGCTGATGGCGTCTCACCACCAACCGATCGAGGTCGATG 1500  
 242 G H K C T I I E A D G V L T K P I E V D 262  
 1501 CGTTTGATATTCAGAGGCCAGAGGTATAGCTGCATCgtaagtctacctatgacctgtt 1560  
 262 A F D I L A G Q R Y S C I 275  
 1561 gtggagataagaacctgactgaatgtatgcgctccaatagTTGAAGGCCGACCAAGATCC 1620  
 275 L K A D Q D P 282  
 1621 TGATTCTACTGGATAAATGCCGCCAATCACAACGTTCTCAACACCAACGTCACAGGCATT 1680  
 282 D S Y W I N A P I T N V L N T N V Q A L 302  
 1681 GCTAGTGTATGAAGATGACAAGCGTCCCTACTACTACCCCTGGAAGCCGTTTGTGACATG 1740  
 302 L V Y E D D K R P T H Y P W K P F L T W 322

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FIG. 2C

```

1741 GAAGATATCAAAATGAATCATTCAGTACTGGCAGCACAAAGCACGGGTCCGACGGTCACAA 1800
322 K I S N E I I Q Y W Q H K H G S H G H K 342

1801 GGGAAAGGGCATCATATAAGTCCGGGCCATTGGAGGTGTATCCGGGTGAGCTCCAG 1860
342 G K G H H K V R A I G G V S G L S S R 362

1861 GGTAAAGACCGCGGAGTGACCTATTCGAAGAAGGCTGTGAGTTGGCTGCTGCACTCGT 1920
349 V K S R A S D L S K K A V E L A A A L V 349

1921 TCGGGTGAGCCGAGTTGGACAAAGAGGCAGAAATGAGGATAATTCGACTATTGTATTGGA 1980
349 A G E A E L D K R Q N E D N S T I V L D 361

1981 TGAGACCAAGCTTATTgttaagtccttaatttttttcgggtgtcacggaagctaaccgcg 2040
361 E T K L I 361

2041 taatagCCGTTGGTTCAACCTGGTGACCGGGGGCTCCAGACCAGCTGACGTGCGTGGTC 2100
361 P L V Q P G A P G G S R P A D V V 379

2101 CCTCTGGACTTTGGCCCTCgtatgtggcttcttgatttcgtccggaatgcaaaactgattt 2160
379 P L D F G L 385

2161 ggggtgggctatagAACTTTGCCAACGGACTGTGGACGATAAACAATGTCTCCTACTCCCC 2220
385 N F A N G L W T I N N V S Y S P 401

2221 TCCGGATGTCCCTACTCTCCTCAAGATCTTGACCGACAAGACAAGTCGACGCTTCTGA 2280
401 P D V P T L L K I L T D K D K V D A S D 421

```

FIG. 2D

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FIG. 2E

2821 AGGCTCTCAGAGTGTCAAGCCTGACGGACAATGGAGAACTATGCGAGAAGTATGAGAA 2880  
536 G S Q S V K P D G Q W K K L C E K Y E K 556  
2881 GTTGCCCTGAAGCACTGCAGTGAAGTTGCAGTTGTTTCCCATTCGGGAAC TGGCTCACTAT 2940  
556 L P E A L Q \* 562  
2941 TCCTTTTGCAATAATTCGGACTTTTATTTTGGGACATTATTGGACTATGCATTGTTTGTC 3000  
3001 ACACCGCGGAAC TAAGCCGAATTC

FIG. 2F

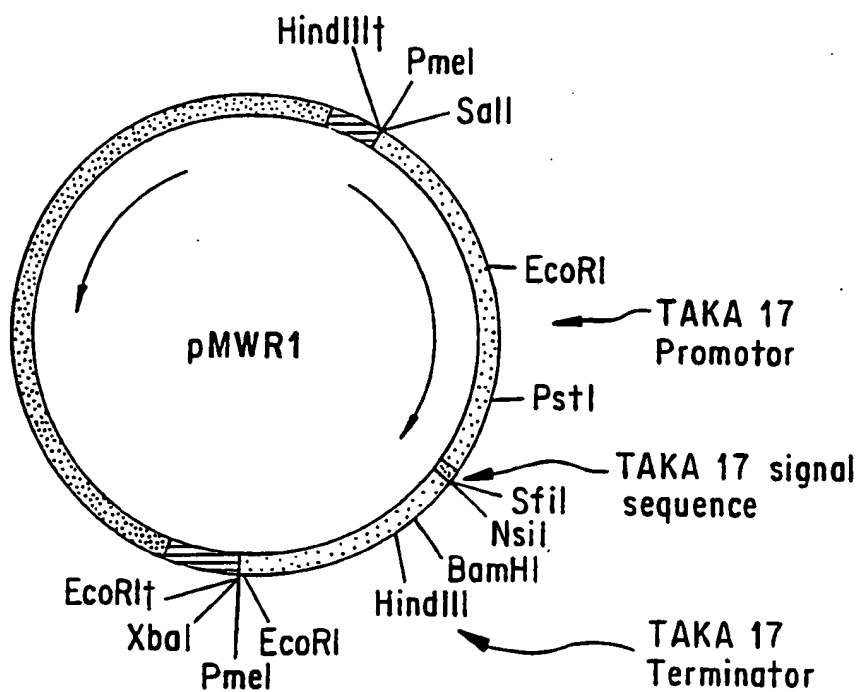


FIG. 3

5' ATG CTT TCT AGC ATT ACC CTC CTA CCT TTG CTC GCT GCG GTC TCA ACC CCC GCC  
 M L S S I T L L P L L A A V S T P A  
 87 96 105 114 123 132  
 141 150 159 168 177 186  
 TTT GCT GCC GTC CGC AAC TAT AAG TTC GAC ATC AAG AAC GTC AAT GTC GCT CCC  
 F A A V R N Y K F D I K N V N V A P  
 195 204 213 222 231 240  
 GAT GGC TTT CAG CGC TCT ATC GTC TCC GTC AAC GGT TTA GTT CCT GGC ACG TTG  
 D G F Q R S I V S V N G L V P G T L  
 249 258 267 276 285 294  
 ATC ACG GCC AAC AAG GGT GAC ACC TTG CGC ATT AAT GTC ACG AAT CAA CTC ACG  
 I T A N K G D T L R I N V T N Q L T  
 303 312 321 330 339 348  
 GAC CCT AGT ATG CGT GCC ACA ACG ATT CAT TGG CAT GGA TTG TTC CAA GCT  
 D P S M R R A T T I H W H G L F Q A

FIG. 4A

357	366	375	384	393	402
ACT ACC GCC GAC GAG GAT GGC CCC GCA TTC GTC ACG CAA TGC CCT ATT GCG CAA					
T T A D E D G P A F V T Q C P I A Q					
411	420	429	438	447	456
AAT TTG TCC TAT ACA TAC GAG ATC CCA TTG CGC GGC CAA ACA GGA ACC ATG TGG					
N L S Y T Y E I P L R G Q T G T M W					
465	474	483	492	501	510
TAT CAC GCC CAT CTT GCG AGT CAA TAT GTC GAT GGA TTG CGA GGC CCT TTG GTC					
Y H A H L A S Q Y V D G L R G P L V					
519	528	537	546	555	564
ATC TAT GAT CCA AAC GAC CCA CAC AAG TCG CGC TAC GAC GTG GAT GAT GCG AGC					
I Y D P N D P H K S R Y D V D D A S					
573	582	591	600	609	618
ACA GTA GTC ATG CTT GAG GAC TGG TAC CAT ACT CCG GCA CCC GTT CTA GAA AAG					
T V V M L E D W Y H T P A P V L E K					

FIG. 4B

CAA	ATG	TTC	TCG	ACT	AAT	AAC	ACC	GCT	CTG	CTC	TCT	654	CCT	GTT	CCG	GAC	TCG	672	GGT	---	---	---
Q	M	F	S	T	N	N	T	A	L	L	S	---	---	---	P	D	S	G	---	---	---	---
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
CTT	ATC	AAT	GGC	AAA	GGG	CGC	TAT	GTG	GGC	GGT	CCC	708	GCA	GTT	CCC	CGG	TCA	726	GTA	---	---	---
L	I	N	G	K	G	R	Y	V	G	G	P	---	---	---	V	R	S	V	---	---	---	---
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
ATC	AAC	GTA	AAA	CGT	GGG	AAA	CGA	TAT	CGC	TTG	CGC	762	GTA	ATC	AAC	GCT	TCT	780	GCT	---	---	---
I	N	V	K	R	G	K	R	Y	R	L	R	---	---	---	I	A	S	A	---	---	---	---
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
ATC	GGG	TCG	TTT	ACC	TTT	TCG	ATC	GAA	GGA	CAT	AGT	816	CTG	ACT	GTC	ATT	GAG	834	GCC	---	---	---
I	G	S	F	T	F	S	I	E	G	H	S	---	---	---	V	I	E	A	---	---	---	---
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
GAT	GGG	ATC	CTG	CAC	CAG	CCC	TTG	GCT	GTT	GAC	AGC	870	TTC	CAG	ATT	TAC	GCT	888	GGA	---	---	---
D	G	I	L	H	Q	P	L	A	V	D	S	---	---	---	I	Y	A	G	---	---	---	---
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

FIG. 4C



897	906	915	924	933	942
CAA CGC TAC TCT GTC ATC GTT GAA GCC AAC CAA ACC GCC AAC TAC TGG ATT					
Q R Y S V I V E A N Q T A A N Y W I					
951	960	969	978	987	996
CGT GCA CCA ATG ACC GTT GCA GGA GCC GGA ACC AAT GCA AAC TTG GAC CCC ACC					
R A P M T V A G A G T N A N L D P T					
1005	1014	1023	1032	1041	1050
AAT GTC TTT GCC GTA TTG CAC TAC GAG GGA GCG CCC AAC GCC GAA CCC ACG ACG					
N V F A V L H Y E G A P N A E P T T					
1059	1068	1077	1086	1095	1104
GAA CAA GGC AGT GCT ATC GGT ACT GCA CTC GTT GAA GAG AAC CTC CAT GCG CTC					
E Q G S A I G T A L V E E N L H A L					
1113	1122	1131	1140	1149	1158
ATC AAC CCT GGC GCT CCG GGC GGC TCC GCT CCC GCA GAC GTT TCC CTC AAT CTT					
I N P G A P G G G S A P A D V S L N L					

FIG. 4D

1167	1176	1185	1194	1203	1212
GCA ATT GGG CGC AGC ACA GTT GAT GGG ATT CTT AGG TTC ACA TTT AAT AAC ATC					
---	---	---	---	---	---
A I G R S T V D G I L R F T F N N I					
1221	1230	1239	1248	1257	1266
AAG TAC GAG GCT CCT TCG TTG CCC ACG CTC TTG AAG ATT TTG GCA AAC AAT GCG					
---	---	---	---	---	---
K Y E A P S L P T L L K I L A N N A					
1275	1284	1293	1302	1311	1320
AGC AAT GAC GCC GAT TTC ACG CCA AAT GAG CAC ACT ATC ATC GTA TTG CCA CAC AAT					
---	---	---	---	---	---
S N D A D F T P N E H T I V L P H N					
1329	1338	1347	1356	1365	1374
AAA GTT ATC GAG CTC AAT ATC ACC GGA GGT GCA GAC CAC CCT ATC CAT CTC CAC					
---	---	---	---	---	---
K V I E L N I T G G A D H P I H L H					
1383	1392	1401	1410	1419	1428
GGC CAT GTG TTT GAT ATC GTC AAA TCA CTC GGT GGT ACC CCG AAC TAT GTC AAC					
---	---	---	---	---	---
G H V F D I V K S L G G T P N Y V N					

FIG. 4E

1437	1446	1455	1464	1473	1482
CCG CCA CGC AGG GAC GTA GTT CGT GTC GGA GGC ACC GGT GTG GTA CTC CGA TTC					
P P R R D V V R V G G T G V V L R F					
1491	1500	1509	1518	1527	1536
AAG ACC GAT AAC CCA GGC CCA TGG TTT GTT CAC TGC CAC ATT GAC TGG CAC TTG					
K T D N P G P W F V H C H I D W H L					
1545	1554	1563	1572	1581	1590
GAG GCT GGG CTC GCA CTT GTC TTT GCC GAG GCC CCC AGC AGC ATT CGC CAG GGT					
E A G L A L V F A E A P S Q I R Q G					
1599	1608	1617	1626	1635	1644
GTC CAG TCG GTC CAG CCC AAC AAT GCC TGG AAC CAG CTC TGC CCC AAG TAC GCG					
V Q S V Q P N N A W N Q L C P K Y A					
1653	1662				
GCT CTT CCT CCC GAT TTG CAG T 3'					
A L P P P D L Q					

FIG. 4F

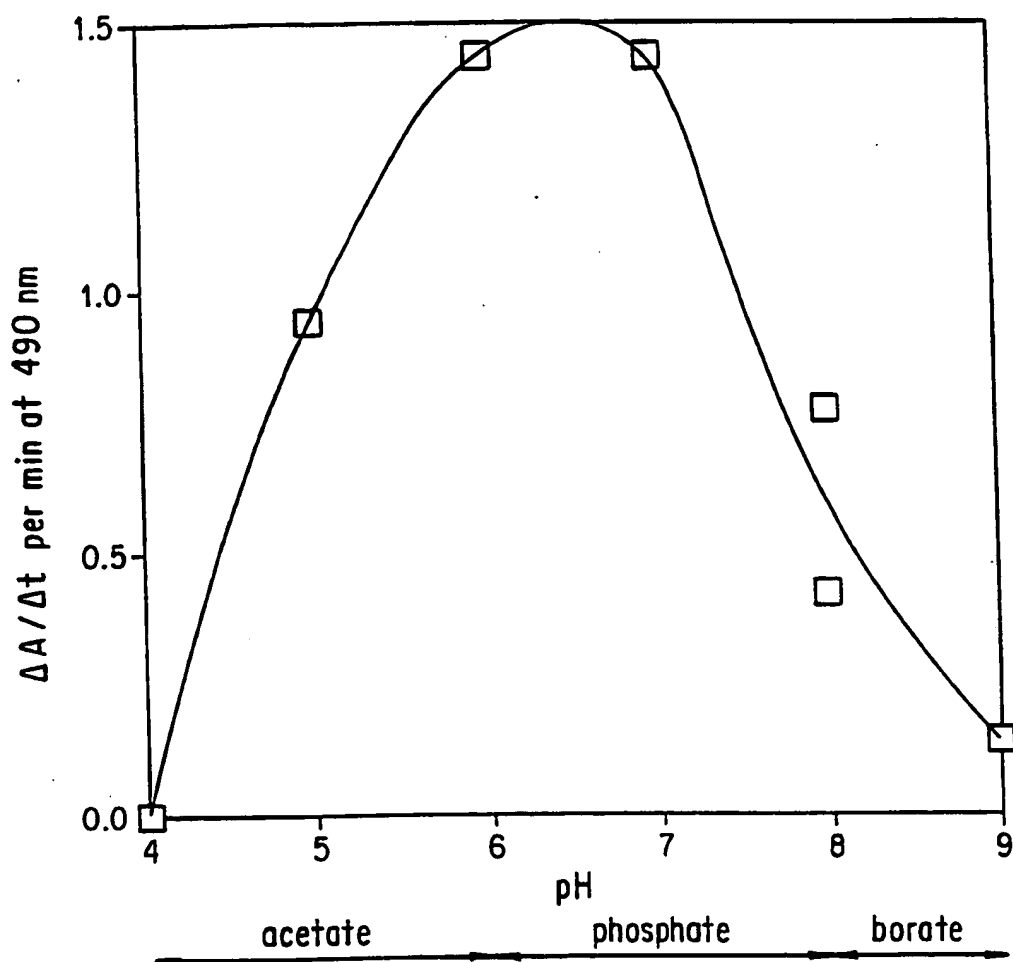


FIG. 5

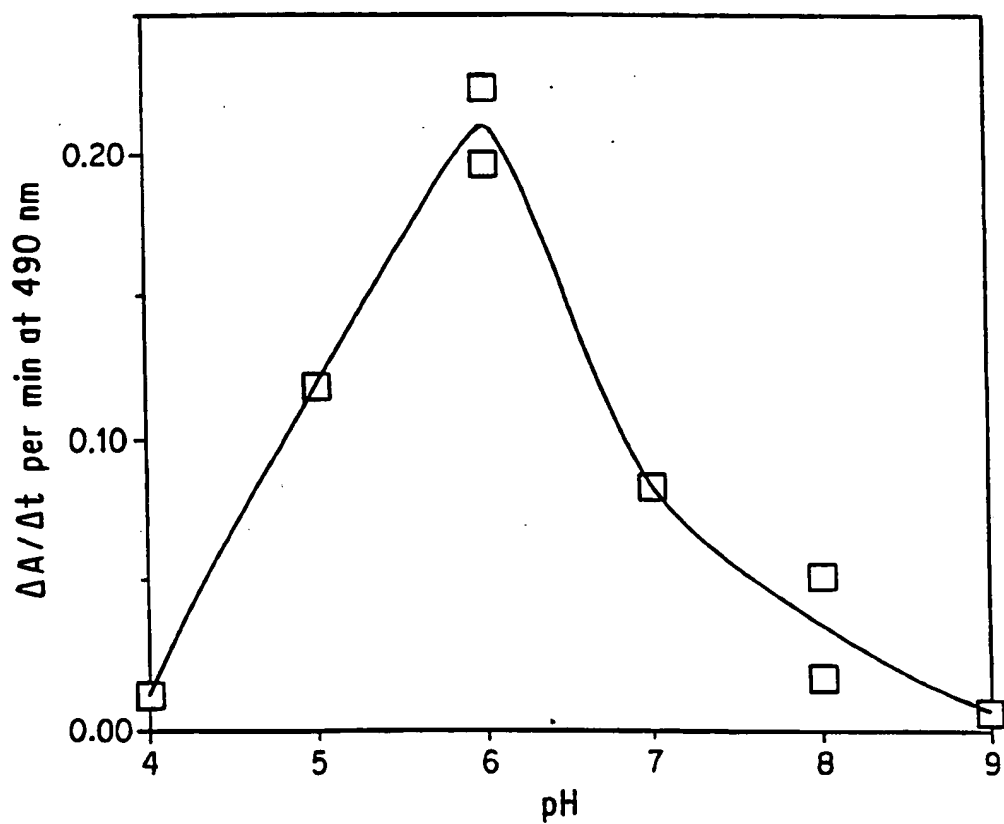


FIG. 6

# INTERNATIONAL SEARCH REPORT

Int: ☐ National Application No

PCT/US 94/10264

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/53 C12N9/02 C12N15/80 D21C5/00 A61K7/06  
C12P7/22 C12N1/19 C09B69/10 //(C12N1/19,C12R1:66)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N D21C A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 90, no. 19, 7 May 1979, Columbus, Ohio, US; abstract no. 147536w, BOLLAG J.M. ET AL. 'Characterization of an enzyme from Rhizoctonia praticola which polymerizes phenolic compounds.' page 213 ; see abstract	14, 43
Y	& CAN. JOURNAL MICROBIOL., vol.25, no.2, 1979 pages 229 - 223 --- -/--	1, 20-24, 39-41

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- \*&\* document member of the same patent family

Date of the actual completion of the international search

24 January 1995

Date of mailing of the international search report

23. 02. 95

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## INTERNATIONAL SEARCH REPORT

Int. Patent Application No

PCT/US 94/10264

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	& ARCH.MICROBIOL., vol.137, no.2, 1984 pages 89 - 96 ---	1,20-24, 39-41
Y	WO,A,92 01046 (VALTION TEKNILLINEN TUTKIMUSKESKUS) 23 January 1992 see claims ---	1,20,21
Y	WO,A,92 16633 (NOVO NORDISK) 1 October 1992 see page 3; claims ---	21-24
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Y	EP,A,0 060 467 (EISENSTEIN) 22 September 1982 see claims ---	41
X	EP,A,0 504 005 (PERMA) 16 September 1992 see claims -----	42

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Information on patent family members

Int: onal Application No

PCT/US 94/10264

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